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A METHOD OF INCUBATING ASCARIS EGGS

BY

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THE eggs of *Ascaris lumbricoides* need three conditions for their development, viz, a moist atmosphere, a temperature about 25°C and clean surroundings They will not develop in water, in dry air, at a low temperature or in a foul medium The conditions needed can be secured by the following simple method —

The eggs are obtained either from the uterus of a fertilized female or by washing out of fæces In the former case the worm is slit open from the gonopore backward, the uteri are pulled out gently and divided into lengths of about two inches The egg-mass is pressed out under water with a bent mounted needle and the contents of one or two sections are transferred with a pipette to a clean test-tube Enough tap-water is added to give about one and a half inches in the bottom of the tube which is then corked and thoroughly shaken so that the egg-mass is broken up, and the eggs float separate in the water The tube is then held horizontally, one end in the fingers of the right hand and the other in those of the left, and rotated slowly and steadily about its long axis so that the water flows slowly round and round over the whole inner surface The eggs sink in the water and stick to the glass so that the inside of the tube becomes coated with a uniform layer of eggs It is the outer albuminous coat which causes the egg to adhere When the greater number have thus adhered the tube is turned upright, the water collects at the lower end leaving the eggs in air which is kept moist by the corking of the tube The tubes are kept at room temperature in the summer or in a low temperature incubator at about 25°C If the contents become at all foul the water should be changed with a pipette Under these conditions the eggs segment and develop steadily until each contains an active embryo Samples can be taken out at intervals on the point of a scalpel and examined in water, and finally the whole cylinder of ripe eggs can be scraped out on the edge of a knife

The method can be adapted for eggs like those of *A mystax* which have no albuminous coat by mixing them thoroughly in water in a small Petri dish, sucking off as much water as possible with a pipette, allowing what remains to evaporate until the eggs are only just moist and then floating the dish in a larger one containing some water This larger dish is then covered with a glass-plate and sealed

ON THE BIOLOGICAL ASSAY OF DIGITALIS BY THE FROG METHOD DESCRIBING A CHARACTERISTIC CURVE FOR *RANA TIGRINA*

BY

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AND

S LAL, M B, B S

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THE frog method for the biological assay of digitalis has not been much favoured in India because of the unsatisfactory results reported by earlier workers such as Chopra and De (1926, 1926a) The conclusions of these workers were based on the use of older methods of biological assay such as the 'one hour frog method' of the United States Pharmacopœia (U S P X) No work by the newer methods introduced by Trevan (1927) for the biological assay of digitalis, strophanthus and squill by the frog method seems to have been done so far in India Trevan, by employing an integrated frequency curve (characteristic curve), was able to minimize the effect of individual variations in the reaction of the test animal This modification introduced by Trevan is, in the words of Burn (1930), already 'transforming the whole subject of biological assay from the plane of an insidious means of self deception to that of a well ordered science' and has been adopted by the 1932 edition of the British Pharmacopœia

The 'characteristic' curves are obtained by injecting groups of animals with different doses of the drug under investigation, noting the percentage mortality at each dose and drawing a smooth curve through the points so obtained The 'sloping' curves owe their shape to the fact that different individuals of a species require different quantities of a given drug to kill them The slope of this 'integrated frequency curve' depends on several factors and amongst other points varies with the species of the test animal and the environment under which it is designed to serve as the test basis For these reasons characteristic curves have been worked for different countries, such as Trevan's for England (*Rana temporaria*), Chapman's for Canada (*Rana pipiens*), Gunn's for South Africa and Behren's for Germany

A similar characteristic curve for *Rana tigrina* which would serve as a test basis in India and particularly in the United Provinces is described below :—

The frog used

Two species of frogs are met with in the neighbourhood of Lucknow—*Rana tigrina* and *Rana cyanophlyctis*—of these, the former is met with much more frequently than the latter, and hence was employed for the working of this characteristic curve   It is the largest of the Indian frogs and often weighs as much as from 150 g to 200 g   The colour is greenish or olive above with dark spots, often a light vertebral line is present   The skin of the back is thrown into longitudinal folds and a strong fold above the tympanum is often present   The male has two subgular vocal sacs, conspicuous externally by the blackish colour of the skin covering them   It is easily distinguishable from *Rana cyanophlyctis* which is brown or olive above, with dark small tubercle-like spots and is also often speckled on its ventral aspect   Frogs weighing between 15 g and 65 g which are easily available were used in obtaining this characteristic curve and the average weight of all frogs used (1,040) was 33 1 g   They were usually obtained fresh from fields for each particular experiment and were not stored in the laboratory reservoir for more than 10 to 20 days   No frogs which had been previously injected were employed in these experiments   Frogs of both sexes were employed   Those obviously diseased, or females swollen with eggs, were discarded

The digitalis preparation

Freshly prepared tincture from British Standard digitalis leaf powder (1 g=10 c c) was used in these experiments   Different dilutions of this tincture were made just before injecting with 0 6 per cent saline solution in tap-water

Experimental method

At first several groups of 20 frogs were injected with varying doses of the tincture to obtain a rough idea of the limits of ' the maximum tolerated dose ' and ' the certainly lethal dose '   Then six doses were selected within these limits and with each of these 20 frogs were injected for obtaining each curve   The frogs were carefully weighed on a sensitive torsion balance by one of us, after drying the frog and expressing its urine   The injections were made with predetermined doses (1 c c of diluted tincture per 100 g of body-weight) into the ventral lymph sac through the thigh muscles   A tuberculin syringe graduated to 0 01 c c was used with a fine long needle   The injected frogs were placed in pairs underneath glass jars, arranged in rows, in a uniformly lighted part of the laboratory, 120 to 160 frogs with six to eight different doses were injected on each day of experiment   One thousand and forty frogs were used for determining the characteristic curve and 140 frogs were used to determine the mortalities on each dose

Results

In Table I are recorded the results of 7 experiments   The dose of tincture of digitalis is a relative dilution percentage , the corresponding per cent mortality is for 20 frogs :—

TABLE I

Date of experiment	Dose in per cent dilution	Per cent mortality	Date of experiment	Dose in per cent dilution	Per cent mortality
A 7-9-1933	25	0	C 18-9-1933	25	0
	30	5		30	10
	35	15		35	35
	40	35		40	45
	45	55		45	65
	50	65		50	95
	55	70		55	85
	60	100		60	100
B 15-9-1933	30	0	D	30	10
	35	5		35	25
	40	15		40	30
	45	55		45	65
	50	55		50	75
	55	80		55	90
	60	90			
E 2-10-33	25	5	F 15-10-1933	30	20
	30	25		35	10
	35	35		40	35
	40	50		45	60
	45	70		50	80
	50	55		55	75
	55	80		60	100
	60	100		65	100
G 15-10-33	25	5		30	10
	35	26		40	35
	45	70		50	100
	55	100			

CHART 1
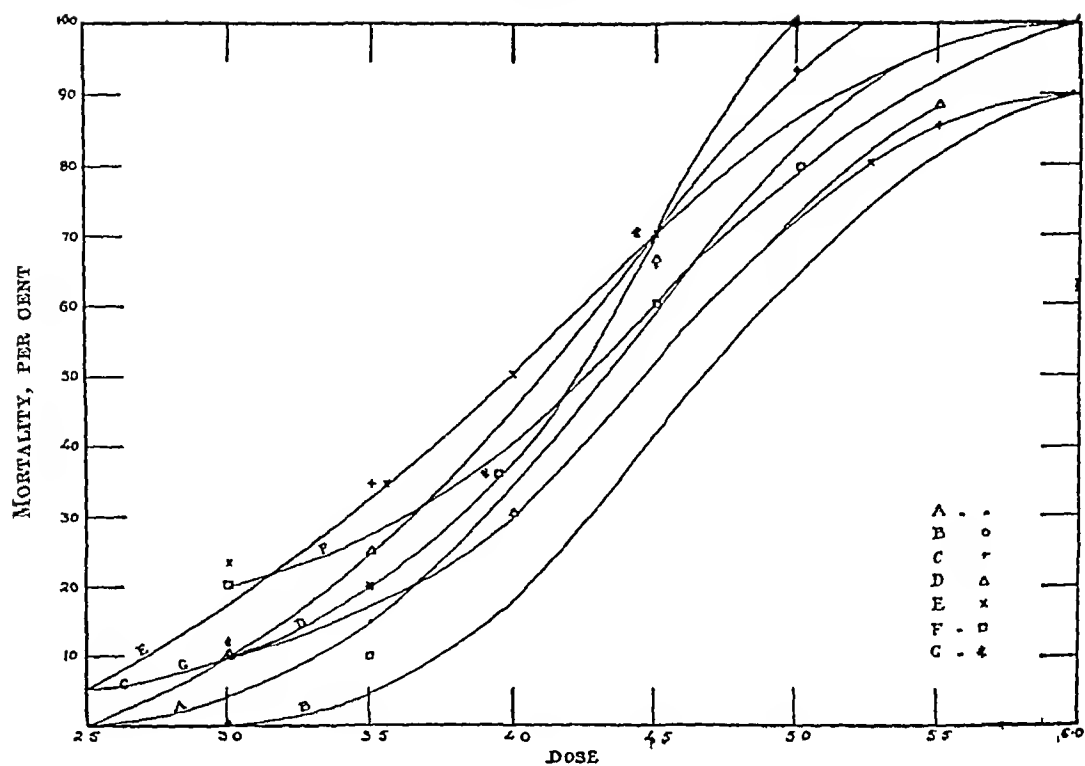
CHART 2
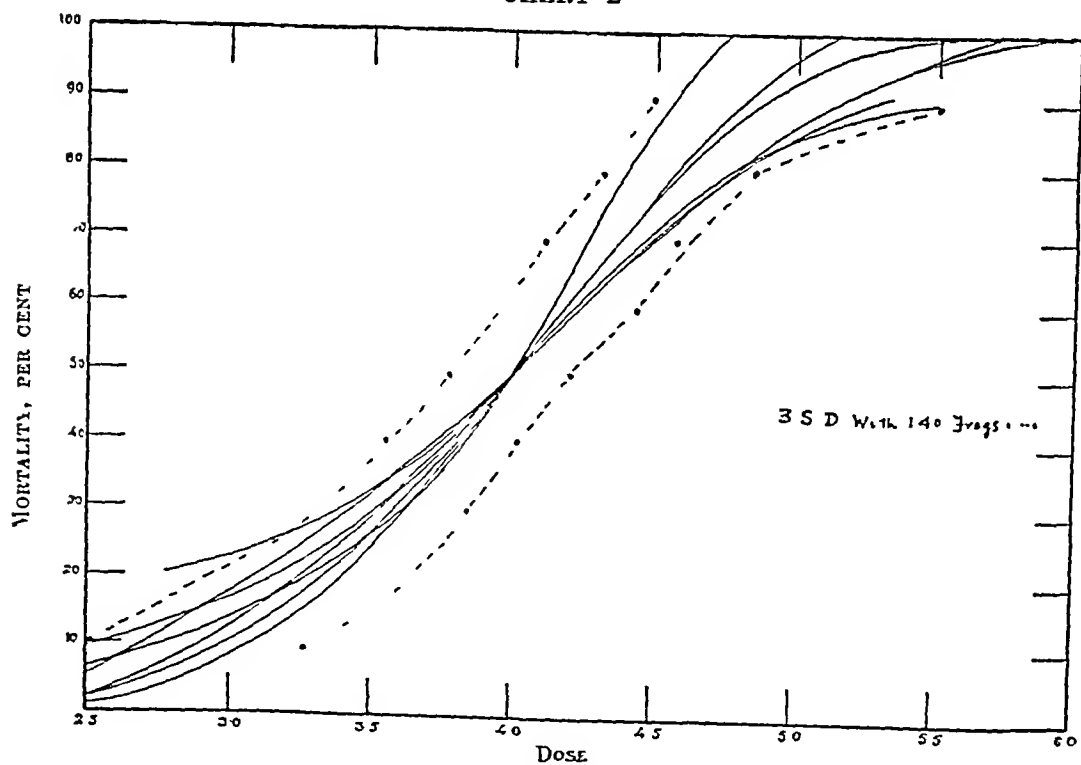
$$\text{Standard deviation (S D)} = \sqrt{\frac{P\,Q}{N}}\ \text{where}$$

P = Percentage mortality   Q = 100—P   N = Number of frogs

Curves corresponding to these 7 experiments are represented in Chart 1   Each curve represents a particular experiment and bears its initial   By choosing a dose value on the abscissal axis corresponding to 50 per cent mortality and moving each curve along the abscissa until its dose value for 50 per cent mortality corresponds to the value selected, the 7 curves were superimposed (Chart 2) without altering their shape   The abscissa selected to represent 50 per cent mortality was given the value of 4   A single curve, representing the combined curve of Chart 2, was constructed and is shown as curve O, in Chart 3   Chart 2 shows

CHART 3
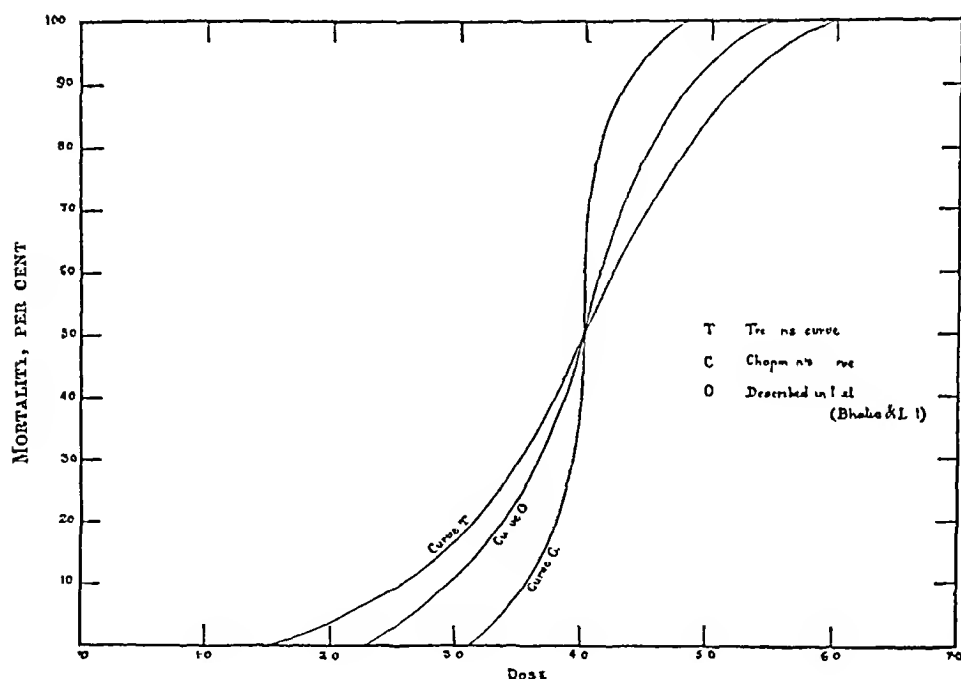
that none of the 7 individual curves vary significantly from curve O, Chart 3   All the seven curves are well within the three times standard deviation limits when 20 frogs are used for each point and almost within the limits of 3 S D when 140 frogs are used for each point   The deviations were measured from curve O, Chart 3

The figures of potency corresponding to a given percentage mortality of frogs are given below in Table II   For the sake of comparison B P figures are also given alongside —

TABLE II

Potency, corresponding to a given percentage mortality of frogs

	POTENCY			POTENCY	
Mortality, per cent	Calculated from B P chart	Calculated from our curve	Mortality, per cent	Calculated from B P chart	Calculated from our curve
5	56	68 5	55	103	102 5
10	67	73 7	60	107	104 5
15	75	78 9	65	110	106 5
20	80	83 2	70	114	108 7
25	83 5	87	75	118	111 2
30	87	90	80	122	113 7
35	90	93	85	127	117 2
40	93 5	95 5	90	134	121 2
45	97	98 2	95	146	126 2
50	100	100			

A perusal of Table II reveals that if the mortalities of a particular experiment are between 15 and 65 per cent, then the difference in calculations based on our curve and B P chart will be within 10 per cent, but if the mortalities are outside these limits, then the figures are likely to be wider apart

TABLE III

Digitalis sample	Original potency	POTENCY WHEN RL ASSAYED		Remarks
		Calculated from our chart	Calculated from B P chart	
Leaf powder L	9 8 units	8 6 units	7 9 units	These samples were originally assayed at the Pharmaceutical Society of Great Britain and were lent by the kind courtesy of Dr J H Burn, the Director
„ „ M	9 7 „	9 1 „	8 4 „	
, , N	16 5 ,	14 9 ,	14 6 „	
Tr R	97 per cent	81 per cent	76 per cent	
Tr P	120 „	90 „	83 „	
Tr T₁	100	107	166 „	The original values of these three tinctures were theoretical values
Tr T	75	69	56	
Tr T₃	138	120	131 ,	

Table III gives results of 8 samples which were either re-assayed by us or their theoretical values (obtained by making different dilutions of a known sample) were compared with experimental findings   Leaf powders L, M and N were sent to us from England in sealed tins   Six to eight weeks elapsed between the original and the second assay   The difference in the figures of original assay and those of re-assay is less than 10 per cent in powders M and N and slightly more than 10 per cent in powder L   Making allowance for a certain amount of deterioration, which must have taken place as the tins were not hermetically sealed, the agreement between the two figures is very close and well within the limits of experimental error   Tinctures R and P were also sent to us from England and periods of 6 and 10 weeks elapsed between the first and second assay   The lower figures obtained by us could easily be due to deterioration which, according to one of us (B B B), is about 10 per cent per month during the first three months   Tinctures T$_1$, T$_2$ and T$_3$ were dilutions of a known tincture made by one of us   The figures of dilution were not revealed to the other author who carried out the assay   The agreement between these figures is also quite close   From these results it is apparent that *Rana tigrina* could be used for assay of digitalis   In nearly all these eight samples the agreement between the original figures and ours is better, when calculations are made from our curve   It is thus claimed that when *Rana tigrina* is used as the test animal calculations made from the curve described by us are likely to be more correct

Discussion

A discussion of the relative merits of the various methods of the biological assay of digitalis will be out of place in a paper like this but undeniably the method of choice will be one which is economical, easy to perform and capable of detecting deterioration   That the frog method meets these requirements better than any other method has been our experience in this laboratory   The cat method of Hatcher and Brody does not detect deterioration and this has a serious drawback in a country like India where deterioration is rapid [about 10 per cent per month during the first three months, according to one of us (B B B)]   It is not economical as for any accurate assay at least 4 to 5 cats would be needed   It takes more time and would involve at least two days' labour on the part of the assaying officer, whereas the injection of 50 frogs takes only about an hour

The modification of the cat method suggested by Chopra and Chowhan (1933) needs further investigation before any definite opinion can be expressed on it   But one fact is very evident that the method as such is capable of only rough estimates of potency for it merely differentiates between a good and a bad sample and does not indicate the exact strength of the given sample   By Chopra and Chowhan's modified method tinctures which are much stronger than the standard will also be allowed to pass on to the market and if prescribed in the usual doses, might lead to toxic symptoms   The new British Pharmacopœia (1932) lays down that a gramme of digitalis leaf should possess an activity of 10 international units and if the powder is more active then it should be mixed either with less active leaf powder or in making a tincture , an allowance is made for the greater activity of the leaf powder by adding sufficient quantity of 70 per cent alcohol to produce a tincture of the required standard strength   This is only possible when the manufacturing chemist is supplied with an exact figure of potency   Any results based on one

containing a thermometer, to the double-surface condenser and then to the double-walled flask wherefrom it is led into the sink The temperature of the water
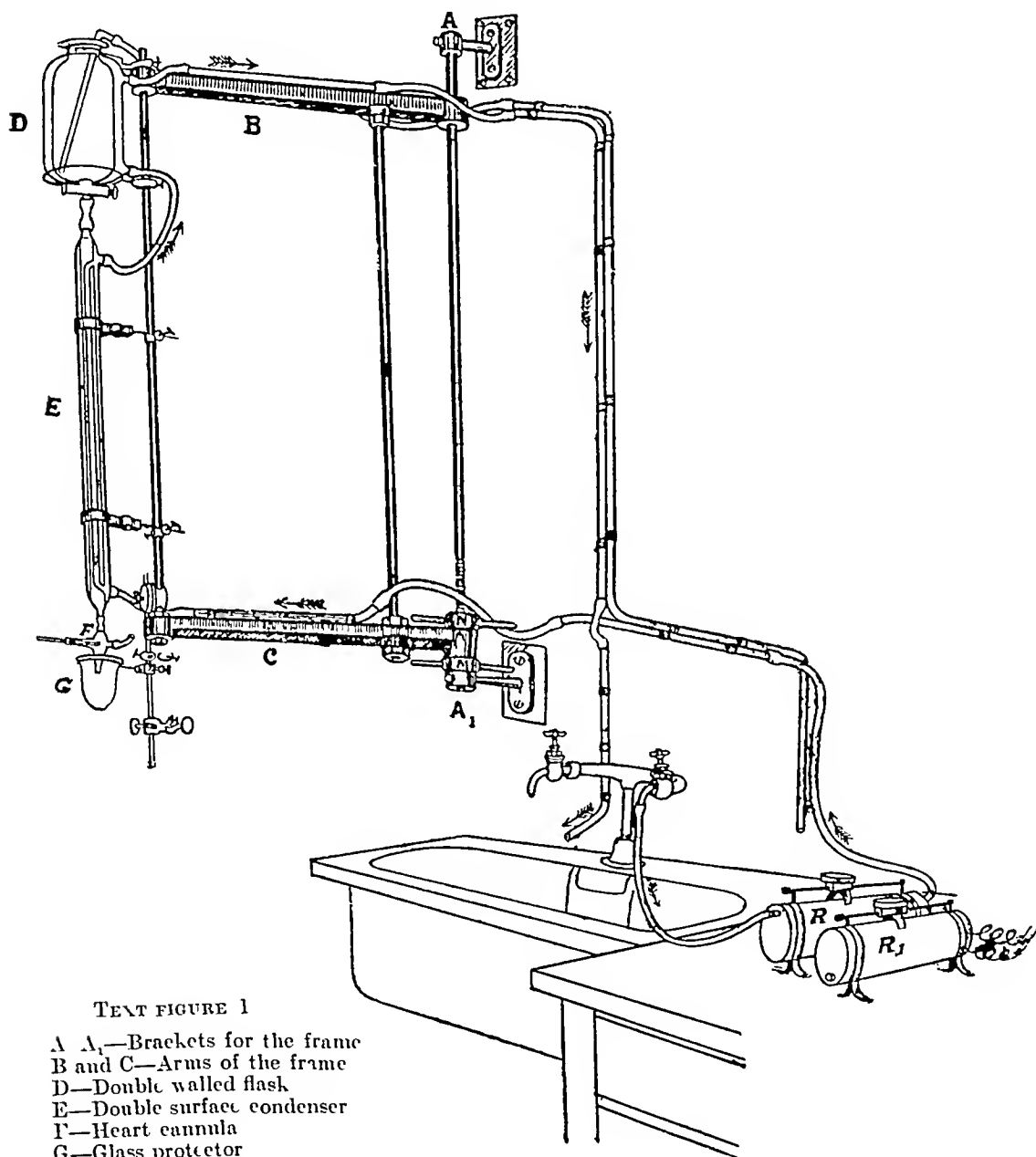
TEXT FIGURE 1

A A₁—Brackets for the frame
B and C—Arms of the frame
D—Double walled flask
E—Double surface condenser
F—Heart cannula
G—Glass protector
M and N—Nuts to move the frame up and down
R, R₁—Rheostats

is adjusted by regulating its flow from the tap and when once adjusted it will remain constant throughout the experiment It is advisable to have a second rheostat R_1

to control the current in the other, as by this means the temperature of the heating system can be adjusted much more easily and rapidly than by regulating the water flow alone   Unnecessary waste of current and water is also eliminated after the apparatus has attained the required temperature   A thermometer inserted into the heart cannula registers the temperature of the perfusing fluid   The apparatus is mounted on a steel frame fixed on the wall and, by manipulating the nuts M and N, it can be moved up and down without disturbing the component parts   The nuts move along screw threads cut on the lower end of the steel rod carrying the frame This arrangement enables records to be taken at different levels of the tracing paper Glass-tubes for water and oxygen run on the wall and reach the apparatus along the arms B and C   When not in use the frame can be folded on to the wall so that the glass parts remain protected from accidents   This contrivance enables space to be saved in a crowded laboratory   A portable type has also been designed to be convenient for transport from one place to another for experimental demonstrations

It is evident from the arrangement of the apparatus that both the flask and the condenser are kept at the same temperature by the current of warm water issuing from the rheostat and it is found that the perfusing fluid attains the same temperature as the water circulating outside, irrespective of the rate of flow of the former   Therefore changes of temperature of the perfusing fluid due to alterations in the heart rate are reduced to the minimum   The apparatus can be brought to the optimum temperature in about five minutes and can be worked continuously without special attention being necessary in regard to control of temperature

CONSTANT TEMPERATURE BATH FOR ISOLATED ORGANS

(Text-figure 2)

This apparatus for perfusing isolated uterus, intestine, etc , consists of a jacketed cylindrical bath of 100 c c capacity A with a three-way stopcock at the bottom and a jacketed two litres flask B, connected together as shown in the diagram   The contrivance for maintaining the perfusion fluid in the flask and the bath at the required temperature is the same as the one employed in the perfusion apparatus for the heart   The jacketed bath may also be attached to the end of the double-surface condenser in the apparatus for perfusing the heart in place of the heart cannula, in which case a separate heating outfit is unnecessary   The tissue is tied to a platinum hook fused at the end of the glass-tube carrying oxygen to the bath   Since the bath and the flask containing the perfusion fluid are heated by the same current of water, they attain the same temperature and, therefore, the fluid in the bath can be changed at any time without varying the temperature, by letting in fresh fluid at the same temperature from the flask by an appropriate turn of the three-way stopcock   After the apparatus has been set for the correct temperature it needs no further attention as to temperature regulation   A number of such perfusion baths can be arranged on a single stand with suitable holders and can be heated to the optimum temperature by the same current of water electrically heated in the rheostat   This arrangement will ensure constancy of temperature in the different baths and will be very convenient for studying comparative effects of drugs under the same conditions and for standardization experiments
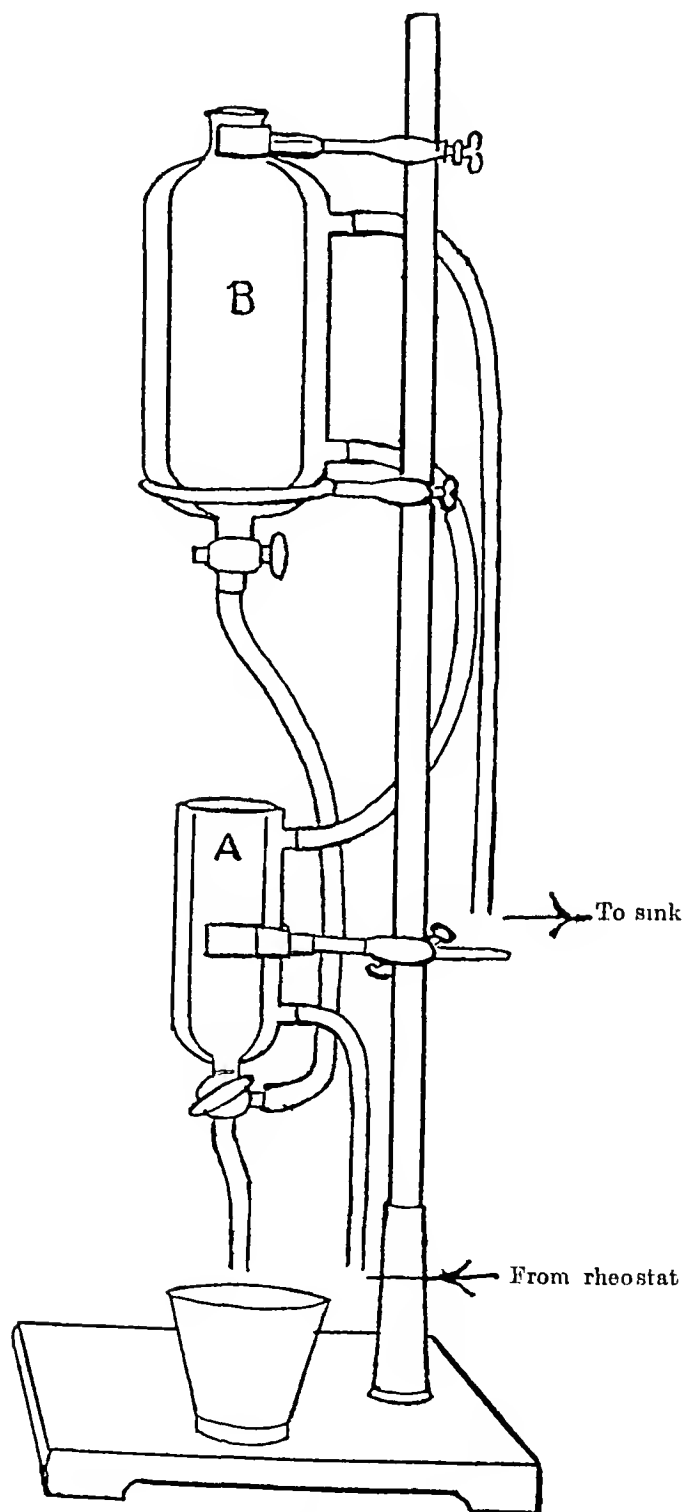
Text Figure 2

Acknowledgments

A DEVICE FOR FILTERING BACTERIOPHAGE

BY

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SINCE April 1932, cholera bacteriophage has been manufactured on a fairly large scale at this Institute for use in field experiments in the prevention and cure of cholera   During the year 1933, about 428,000 c c of cholera 'phage were issued   It has been found convenient to distribute it in 20 c c ampoules   Apart from difficulties encountered in obtaining a potent 'phage, which depends for its potency on various factors that need not be gone into here, it was found that serious difficulties arose during the process of filtration by way of contaminations, candles found cracked during sterilization, etc   A method of filtration has been evolved which has worked so successfully during the past year with a minimum of waste in labour and material, that it would be useful to describe it   Sixty litres of 'phage have been handled weekly without overstraining the limited staff of the section   A brief description of the apparatus follows —

The diagram shows the apparatus assembled during filtration   The fluid that is to be filtered is contained in the glass-jar in which a candle—Pasteur-Chamberland ' F '—is dipping, filtration through the candle being from without inwards   The side tube of the filtering flask is connected to the vacuum line and a partial vacuum is maintained about the neighbourhood of 15″ of mercury   The bent glass-tube *a*, one end of which reaches the bottom of the filter-flask, serves the main purpose of emptying out later, the contents of the flask into Maynard ampoules , but it also serves as an indicator of the pressure inside   When pressure in the flask increases as a result of leakage into it of outside air the fluid rises in the tube and gives a warning that the vacuumizing plant is to be switched on

As filtration proceeds, the fluid in the glass-jar diminishes and ordinarily fresh fluid would have to be poured into it so as to maintain the maximum rate of filtration   To ensure a constant level of fluid in the jar, an apparatus L has been devised somewhat on the lines of Gardner (1931) for his serological water-bath   It consists of a 1 5 litre flask fitted up in the fashion of a wash-bottle, except that both the tubes reach its bottom   The tube S serves as a syphon   To the outer end of the tube S is attached one end of a piece of rubber-tubing, the other end being kept dipped in the glass-jar by the side of the candle   A screw pinch-cock is fixed

about the middle of the tubing   The second tube R serves two purposes—first to start the syphon and, secondly, to maintain a constant level of fluid in the glass-jar   To work the apparatus the flask is filled to the neck with the fluid to be filtered and the rubber-bung is fitted on tightly   The syphon is now started by opening the pinch-cock P and blowing through the tube R at M   It will be seen from the diagram that the flask L is placed on the bench at a conveniently higher level   The fluid in the flask will flow through the syphon into the glass-jar until the level in the latter comes up to that of the open end O of tube R , no further rise in the level in the jar will take place   As it falls with the progress of filtration, more fluid will flow over from flask L and its place

DIAGRAM
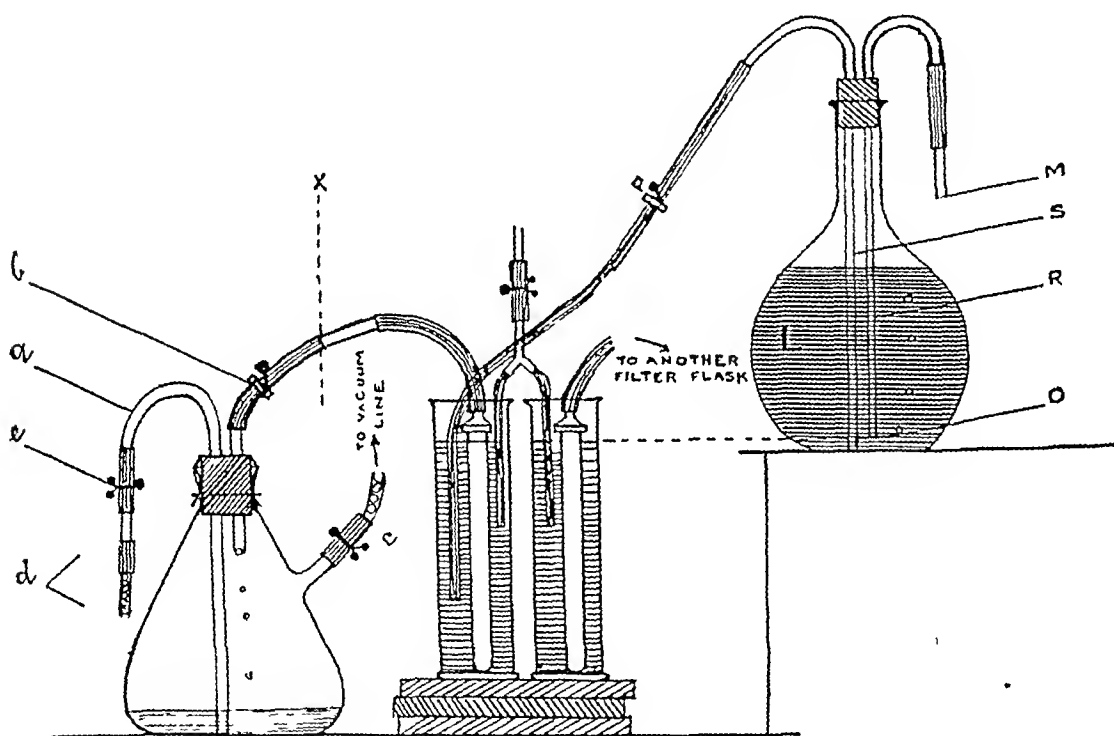
will be taken by air bubbling through the open end O of tube R   Under static conditions, the surface of fluid in the jar will be on a level with O, but during filtration the latter is usually found to be at a slightly lower level depending on the rapidity of filtration   The fluid outside should not rise beyond the glazed shoulder of the candle and it is preferable to have the level adjusted to about a quarter of an inch below the shoulder   This is done by placing thin blocks of wood underneath the jar

When the fluid in flask L has gone down, it can easily be charged again with more fluid without disturbing the syphon   The pinch-cock P is screwed in tightly the mouth of the flask is opened and more fluid poured in   The cork is refitted, the pinch-cock opened and the syphon begins to work, the level being maintained as before

As each of our 'phage brews is 2 5 litres in bulk and this is too much for a candle, two candles are employed working side by side   The two jars containing the candles are connected up by a Y-tube as shown in the diagram   By this arrangement the same level is maintained in both the jars   In fact, with a single levelling apparatus described above, it is possible to have a battery of candles working at the same time

When filtration is over, the rubber lead from the filter-flask is pinched with a screw pinch-cock at *b*, and it is disconnected from the candle   As a precaution against contamination the loose end of the rubber tube is flamed and introduced into a sterile test-tube which is then plugged   The partial vacuum in the filter-flask is next reduced to zero by allowing filtered air to pass into it through the side opening *c* of the flask

The flask is incubated for 24 hours   This period has been found to be sufficient If the fluid keeps clear and limpid it is ready for filling into Maynard ampoules The portion *d* is disconnected the rubber attachment of the Maynard filler is fitted on to the glass tube the pinch-cock *c* is opened and the vacuumized ampoules are filled by the usual method   The sealed ampoules are incubated for 72 hours for ' sterility '

Mention must be made of the following few points —

1   It has been found to be convenient to autoclave the filter-flask and the candle separately (separated by line X in the diagram) and connect them up just before filtration

2   The attachments of the levelling-flask, the Y-tubes and the glass-jars are sterilized by boiling in water for half an hour

3   Each flask has its distinctive number etched on its neck and the same number is marked on the corresponding rubber-bung, so that after cleaning the assembly is facilitated   This serves a useful purpose as will be seen below

4   A book is maintained giving the number of the 'phage brew and against it the numbers of the two flasks and against each of these again that of the candle, thus Brew 358—$\frac{F1\ 8}{52}$, $\frac{F1\ 9}{64}$ Very rarely it happens that contamination occurs as a result of defects in the flask fittings   If a particular filtrate has been found contaminated, and the candle used has been found to be sound, suspicion is naturally aroused about the flask and its fittings and any defects therein can immediately be attended to   A square piece of water-proof sheeting going over the rubber-bung of the filter-flask, pierced in two places to take the tubes and secured round the neck of the flask with a piece of string, has been found to be of use as a guard against contamination   It stands repeated sterilization very well

REFERENCE

GARDNER, A D (1931)   ,   ' A System of Bacteriology ' **9**, p 188

STRUCTURE AND SIGNIFICANCE OF THE HYPOPHYSIO-PORTAL SYSTEM *

BY

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AND

D V S REDDY, M B, B S

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THE internal circulation of blood in the pituitary body and *tuber cinereum*, in the dog was described by one of us (Basir, 1932) in an earlier paper   Our findings in the dog agree to a remarkable extent with those of Popa and Fielding (1930, 1930a) who are working on the human pituitary, excepting a few minor differences dependent on the anatomical peculiarities of the human pituitary

The pituitary body receives its arterial blood-supply from the branches of the circle of Willis and from the internal carotid arteries   These vessels form irregular sinusoids in the *pars anterior* and *pars tuberalis* which later communicate with the capillary plexus of the *pars nervosa*   The latter also receives direct blood-supply from the posterior lobe artery   Finally, the venous blood is drained partly into the *tuber cinereum* through a series of short parallel vessels, the hypophysio-portal vessels

The nuclei of the *substantia grisea* of the *tuber cinereum* get their blood-supply from the hypophysio-portal vessels besides arterial blood derived directly from the systemic vessels of the circle of Willis   Blood vessels from both these sources form a well-marked secondary capillary network amongst the neurones of the various

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* After this paper was sent for publication, we have come across an interesting contribution on ' The Development of the Hypophysio Portal System in Man', by Paul G'Espinasse, in *Jour Anat* (1933), **68**, p 10, wherein the author agrees with the earlier findings of Basir (1932) regarding the absence of the spaces of Virchow-Robin, around the hypophysio portal vessels   He is of opinion that the hypo physio portal vessels, which are part of the arterial supply of the brain, starting from the pia mater, have become secondarily involved by the up growing buccal analogues of the pituitary body   Here, they lose their arachnoidal sheaths which surround the spaces of Virchow Robin   We have again confirmed this fact by the more elaborate methods of injections into the sub arachnoid space

nuclei of the diencephalon and also under the ependyma of the infundibular recess

In an earlier paper the senior author (Basir, 1932) has mentioned that this dual blood-supply to the tuberal nuclei can be differentiated microscopically by the fact that the systemic vessels are enclosed in a perivascular space—the space of Virchow-Robin—which cannot be made out round the vessels of the hypophysio-portal system

As this observation is an important one, Professor J P Hill, F R S , of University College, London, suggested to the senior author to have it confirmed by experimental methods   Consequently the present investigation was undertaken in our laboratory   The experimental methods used in this investigation are more or less on a line with the technique adopted by Weed (1914) in his studies on the cerebrospinal fluid

TECHNIQUE

Dogs were first anæsthetized by ether and then chloralose (0 1 g per kilo body-weight) was injected into the small intestines   In order to know the condition of the cardiovascular system during the experiments, the blood-pressure of the animal was recorded by introducing a cannula into one of the carotid arteries   Then the dog was placed in the prone position and laminectomy of the lower thoracic or upper lumbar vertebræ was done   The spinal cord was then pulled out of the vertebral canal without injury to the meningeal covering of the nerve roots

Transection of the spinal cord was performed and about half an inch of the cord with the pial covering was excised leaving behind the dura mater and the arachnoid uninjured in the form of a hollow sleeve-like cuff into which a cannula was tied   This cannula was then connected to an especially devised apparatus (Text-fig 1) containing a fluid which could be injected intrathecally at any desired pressure varying from 150 mm of water to 150 mm of mercury   During the experiment care was taken to avoid air bubbles and leakage from the dural cuff   The temperature of the fluid was maintained at 40°C throughout the experiment

The following three different kinds of fluids were injected intrathecally in a series of experiments —

(1) *Injections of granular suspension of Indian ink* —Weak suspension of Indian ink was filtered through a coarse filter-paper and then injected intrathecally at a pressure of 60 mm Hg for 6 hours   This attempt was a failure and the Indian ink was only found superficially in the leptomeninges of the pituitary body

(2) *Injections of milk* —Diluted milk was injected similarly at a pressure of 35 mm Hg for a duration of 4 hours   The tissue was subsequently fixed in 5 per cent formol in isotonic saline and embedded in gelatin according to the method of Gaskell and Graff as modified by Nicolas and sections of 25 μ were cut by means of a freezing microtome   After staining the sections with Scarlach R and Delafield's hæmatoxylin they were mounted in dilute glycerine and examined microscopically Unfortunately the milk was seen not to have penetrated the tissue well and the results were not quite satisfactory

(3) *Injections of true solutions* — (a) A solution containing potassium ferro-cyanide (1 per cent) and citrate of iron (1 per cent) was made in normal saline

and injected intrathecally at 40°C for 6 hours at a pressure of 150 mm of water. Throughout the experiment the solution flowed into the sub-arachnoid space at the rate of 9 drops per minute. The blood-pressure of the animals during these experiments fell to zero within about an hour after the beginning of the injections. Finally, a dilute solution of hydrochloric acid (1 per cent) with or without formalin (10 per cent) was injected into one or both carotids by a special two-way syringe. The formalin fixed the tissue and the acid gave a precipitate of Prussian blue when it came in contact with potassium ferrocyanide. The pituitary with a part of the *tuber cinereum* was then dissected out and fixed in 40 per cent formol containing 1 per cent hydrochloric acid.
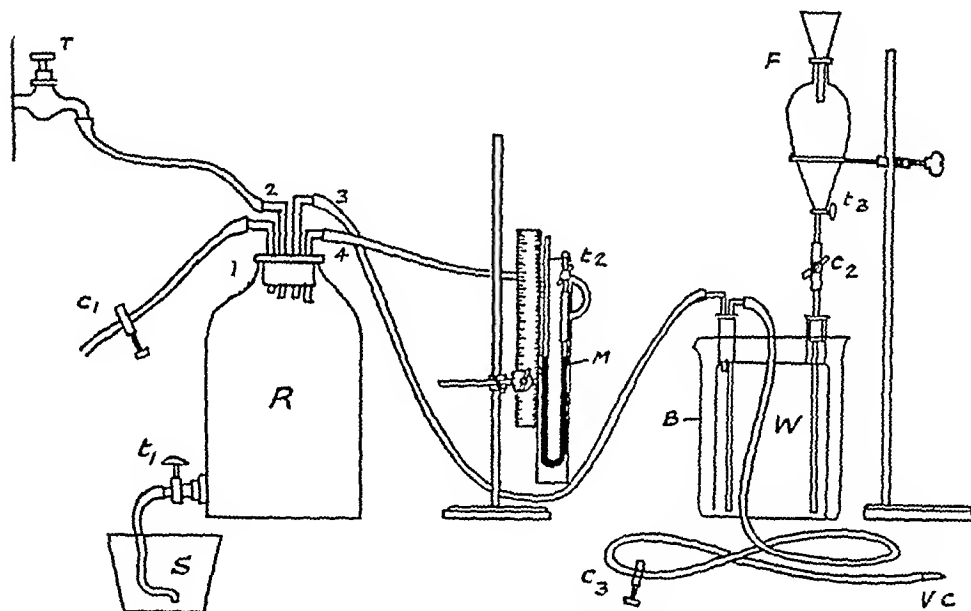
TEXT FIGURE 1 —Diagram of the apparatus for intrathecal injections

The water from the tap (T), flows through the tube (2) into the air tight reservoir (R) and raises the pressure within. This pressure may be measured by the manometer (M) which is connected to the reservoir (R) through the tube (4). The tube (3) transmits the same pressure to the Wolf's bottle (W) containing the fluid, for intrathecal injections. The pressure with which the injection is to be done may also be modified by the tap (t_2) attached to one of the limbs of the manometer (M). On releasing the clip (C_3) the fluid from the Wolf's bottle (W) will flow out through the venous cannula (V C) which is tied to the dural cuff. The temperature of the fluid is kept at 40°C by immersing the Wolf's bottle (W) in a water bath (B). The tap (t_3) and clip (C_2) will put the Wolf's bottle (W) in communication with funnel (F) through which it may be filled. The tube (1) of the reservoir (R) bearing the clip (C_1) and the tap (t_1) are for emptying the reservoir (R) and the waste water will then flow into the sink (S). It is essential that the whole apparatus is air tight.

(b) In another series of experiments, a 10 per cent solution of potassium ferrocyanide alone was injected similarly at a pressure of 60 mm Hg. But here the animal died soon after the beginning of the injection and the fluid entered the

sub-arachnoid space at a rate of 55 drops per minute   At the end of 4 hours, a 1 per cent solution of hydrochloric acid was injected through both the carotids by means of a special syringe   The pituitary body and the *tuber cinereum* were similarly fixed in 40 per cent formol containing 1 per cent hydrochloric acid

The tissue was embedded in paraffin and serial sections of 20 μ thickness were cut and stained lightly with Ehrlich's or Weigert's hæmatoxylin and eosin, and studied under the microscope

OBSERVATIONS

The hypophysis is completely invested by the leptomeninges with a definite sub-arachnoid space as seen by the distribution of the Prussian-blue granules   The pigment is seen along the perivascular spaces in the stroma of the *pars anterior* and *pars tuberalis* where the intimate connection between the blood and the cerebrospinal fluid becomes apparent   The blue granules can also be traced into the *pars intermedia* and *pars tuberalis*   The capillary loops of the infundibulum are also surrounded by the granules of Prussian blue   But when these vessels are traced up in the stalk and *tuber cinereum* (which are here known as hypophysio-portal vessels) the pigment becomes markedly decreased and even completely disappears (Plate I, fig 1)   In contrast to this, the systemic vessels and their branches are heavily surrounded by the pigment (Plate I, fig 2)   In the various nuclei of the *substantia grisea* of the *tuber cinereum*, the pigment is seen to be irregularly distributed on account of the free anastomosis of the systemic and the hypophysio-portal vessels   Consequently, the various nuclei stand out prominently surrounded by areas with little or no pigment granules of Prussian blue

DISCUSSION

The hypophysio-portal vessels are characterized by the following structural peculiarities —

(1) The hypophysio-portal vessels can be readily distinguished from the systemic vessels by the absence of the Virchow-Robin space which is seen clearly to surround the systemic vessels (*cf* Plate I, figs 1 and 2)

(2) The hypophysio-portal vessels are surrounded by sheaths of glial tissue, whilst systemic vessels do not possess such glial sheaths (Basu, 1932)

(3) The walls of the hypophysio-portal vessels contain no muscular tissue (Basu, 1932)

From these structural peculiarities of the two systems of blood vessels, it can be easily surmised that the masses of the colloid of the posterior lobe of the pituitary body, find their way more readily into the blood stream of the hypophysio-portal vessels than that of the systemic vessels which are surrounded by the Virchow-Robin space containing the cerebrospinal fluid   In the latter system of blood vessels the colloid masses get themselves dissolved in the cerebrospinal fluid and very little enters the actual blood stream   Consequently, the blood of the hypophysio-portal vessels should contain more of the hormones of the pituitary body than the blood of the systemic vessels

PLATE I
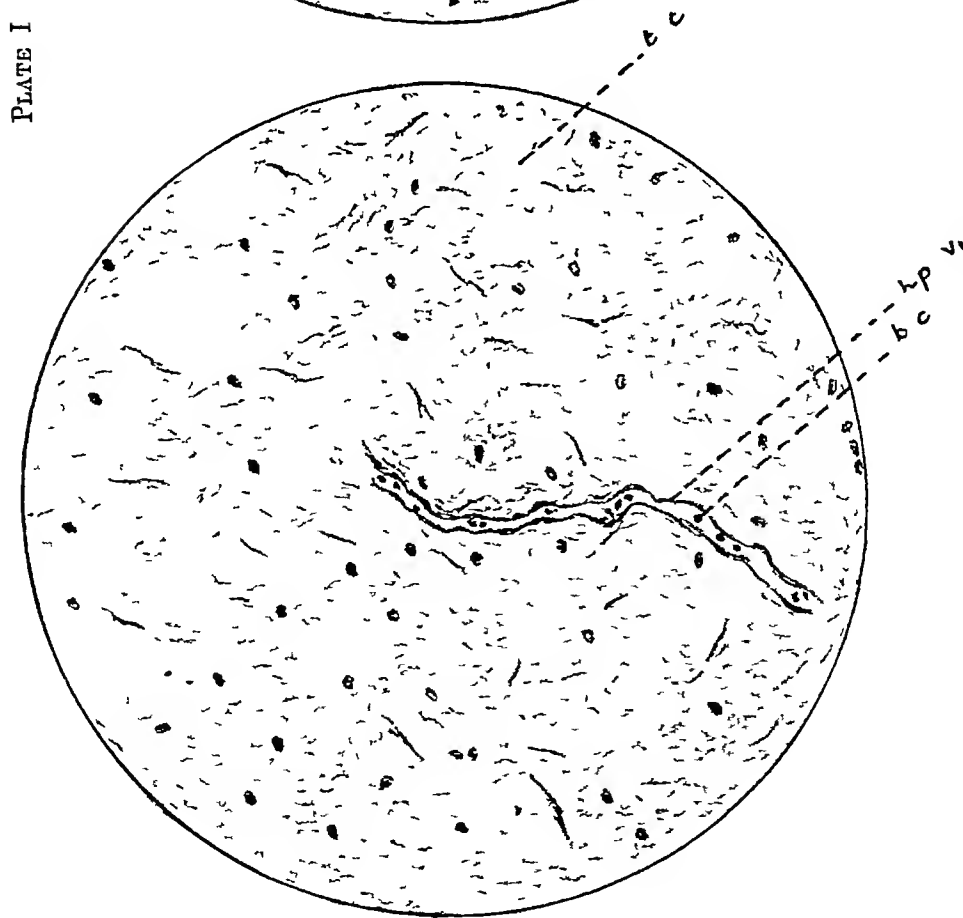
Fig 1 —High power drawing of photomicrograph of the *tuber cinereum* showing a hypophysio portal vessel

Sagittal section   Dog   Mag   ×200   Note the long   hypophysio portal   vessel (hp V₁) passing through the *tuber cinereum* (t c), containing corpuscles (b c) in its lumen   Note the absence of the Prussian blue granules outside the vessel, indicating the absence of the Virchow Robin space around it
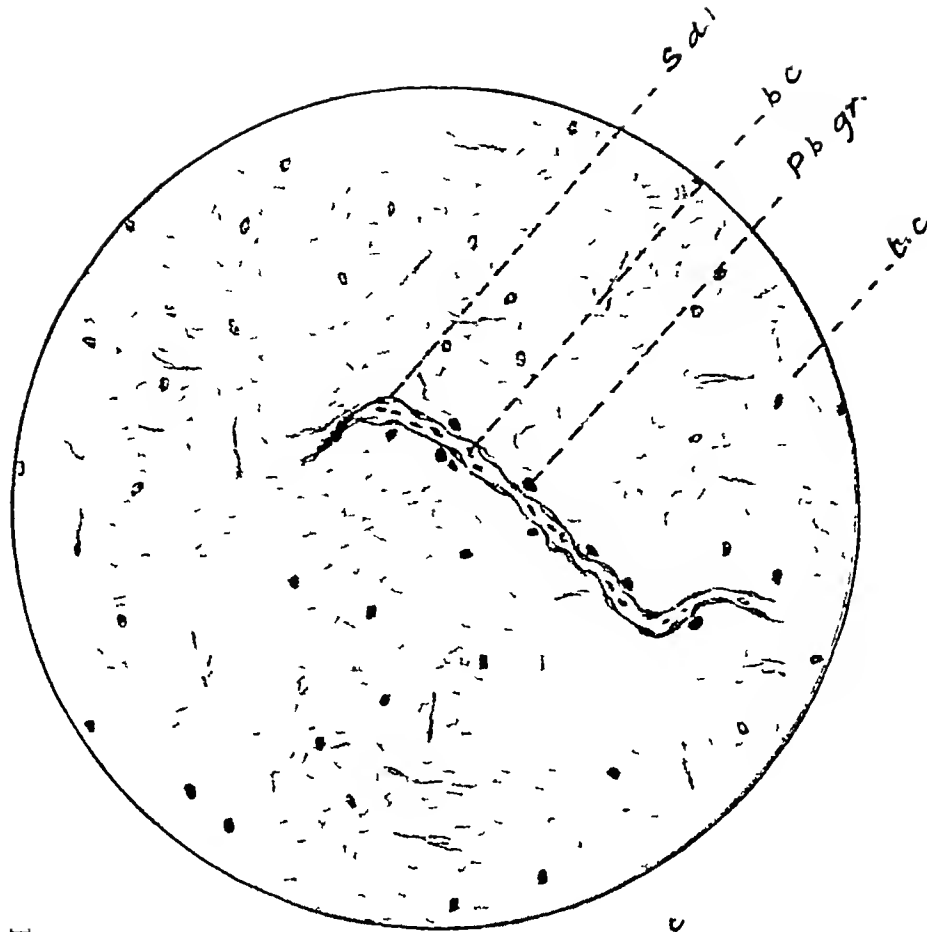
Fig 2 —High power drawing of photomicrograph of *tuber cinereum*, showing a systemic vessel

Sagittal section   Dog   Mag   ×200   Note the long systemic vessel (S a₁) passing through the *tuber cinereum* (t c)   The blood vessel contains corpuscles inside and granules of Prussian blue (P b gr) outside the lumen, lying in the Virchow Robin space, which is very narrow around such a minute vessel

The vascular route of absorption of the hormone of the posterior lobe has already been demonstrated beyond doubt by Hogben (1924) in amphibia and by Krogh (1926) in his Amnia melanophore preparations which show that pituitrin is present in greater concentration in the equine serum than in the equine cerebrospinal fluid   Again, McLean (1928) states that the amount of pituitrin as measured by the oxytocic method is 180 μ mg in the external jugular vein, 120 μ mg in the carotid and 120 μ mg in cerebrospinal fluid, showing thereby that pituitrin is carried to a larger extent by the plasma of the venous blood than the cerebrospinal fluid

Although pituitrin is carried largely by the blood stream, we did not find masses of colloid in the lumen of the hypophysio-portal vessels, whilst the intra-neural colloid could be readily seen after staining with brazilin   But Rasmussen (1927) and more recently Popa and Fielding (1933) have described masses of granular secretion in the blood stream of these vessels   Our finding, therefore, is in agreement with that of Herring (1914-15) and others, who have noticed the absence of masses of the colloid in the lumen of these blood vessels   The absence of the colloid masses in the blood stream is perhaps due to the solubility of the hormone in the plasma of the blood

Since the diencephalic nuclei (Basir, 1932) are richly provided with these hormone-laden hypophysio-portal vessels, it makes it possible that the hormone exercises a local effect, perhaps stimulatory, on these nuclei   The cells of these nuclei appear very similar to the cells of the sympathetic system   Kappers (1933) has found them to be more or less chromaffin cells and to possess sometimes more than one nucleus   It is in these sympathetic cells of the diencephalic nuclei that there exists the wonderful mechanism of vegetative instincts, e g , alimentary instinct in satisfying hunger and thirst, recovery from fatigue by sleep, the regulation of body temperature and metabolism and the sex instincts by which there is preservation of the species, etc   So all these emotional and vegetative reactions are integrated in these diencephalic nuclei in the neighbourhood of which the hypophysio-portal vessels appear to disgorge their hormone   Such local and stimulatory effects of the hormones of the pituitary body on the sympathetic nuclei of the diencephalon can be compared to the well-known similar action of adrenalin on the sympathetic nervous system   One perhaps acts as an adjuvant to the other   This fact is well borne out by the recent experiments of Melville (1932) who showed that injection of smaller doses of adrenalin and pituitrin together is more effective than larger individual doses of these hormones in combating respiratory and circulatory failures

Greving (1923) has stated that these nuclei which lie under the wall of the third ventricle send non-medullated nerve fibres through the stalk in the form of compact bundles which run into the *pars nervosa* and ramify in ' basket-like ' fashion around the cells of the *pars intermedia*   Croll (1928) also has given a similar description ' These nerve fibres are not vasomotor in function as the *pars nervosa* has a poorer blood-supply than the *pars anterior* which gets fewer nerve fibres although richly supplied by blood vessels '   This anatomical distribution of the non-medullated nerve fibres from the diencephalic nuclei to all the parts of the pituitary body, and to a greater extent to the posterior lobe, presumably shows that these primitive vegetative nuclei also control the output of the hormone of the pituitary body particularly that of the posterior lobe

Conclusion

Since the diencephalic nuclei are richly supplied with the hormone-laden hypophysio-portal vessels, it is possible that this hormone exercises a local effect of stimulation of these nuclei   This is partly proved by the recent experiments of Raab (1930) who showed that the normal activity of the fat-regulating centre in the hypothalamus is dependent on the adequate supply of pituitrin

The distribution of the non-medullated nerve fibres from these diencephalic nuclei to the pituitary body suggests that there is a mechanism of reciprocal inter-action between the pituitary body and the vegetative nuclei of this primitive portion of the diencephalon   These nuclei are, therefore, in a position to regulate the libera-tion of the hormones of the pituitary body which are necessary for their optimal reactions

Moreover, it should be remembered that the hypothalamus which has come to be looked upon as the chief head ganglion of the sympathetic system, sends many non-medullated nerve fibres by way of the spinal cord to the thoracic sympathetic system   Karplus and Kreidl (1914) have experimentally demonstrated its influence upon blood-pressure and the pupils through the thoracic sympathetic fibres   Again, according to Hollander (1931), certain regions of the parietal lobe preside over activities of the sympathetic system   There is also enough physiological evidence to show that to a certain extent the hypothalamus is under cortical control, even though no direct tracts are known to link them together

The neuro-hypophysial mechanism is thus a very extensive one playing an important rôle in the normal vegetative functions of the organism   Text-fig 2 is a schematic representation of these inter-relationships   It should also be remembered that other endocrine glands which are closely inter-related to the pituitary body may indirectly exert their influence on this neuro-hypophysial mechanism.

Summary

(1) The vascular supply of the pituitary body is briefly described

(2) The absence of Virchow-Robin spaces around the hypophysio-portal vessels is experimentally shown by injecting intrathecally into the lumbar region of the spinal cord solutions of potassium ferrocyanide and citrate of iron and fixing the tissues in acid-formalin   A deposit of Prussian-blue pigment granules is noticed around the systemic vessels which are surrounded by a Virchow-Robin space whilst no such deposit is seen around the hypophysio-portal vessels which are devoid of the perivascular space

(3) Intrathecal injections of Indian ink and milk have not yielded satisfactory results

(4) Since the diencephalic nuclei are richly supplied by the hormone-laden hypophysio-portal vessels, it is surmised that pituitrin exercises a local stimulating effect on these vegetative nuclei

(5) Since non-medullated nerve fibres proceed from the diencephalic nuclei to the pituitary body it is believed that there is a mechanism of reciprocal interaction between the pituitary body and the primitive diencephalic nuclei which possibly regulates the liberation of the hormone of the pituitary body, necessary for their optimal reactions
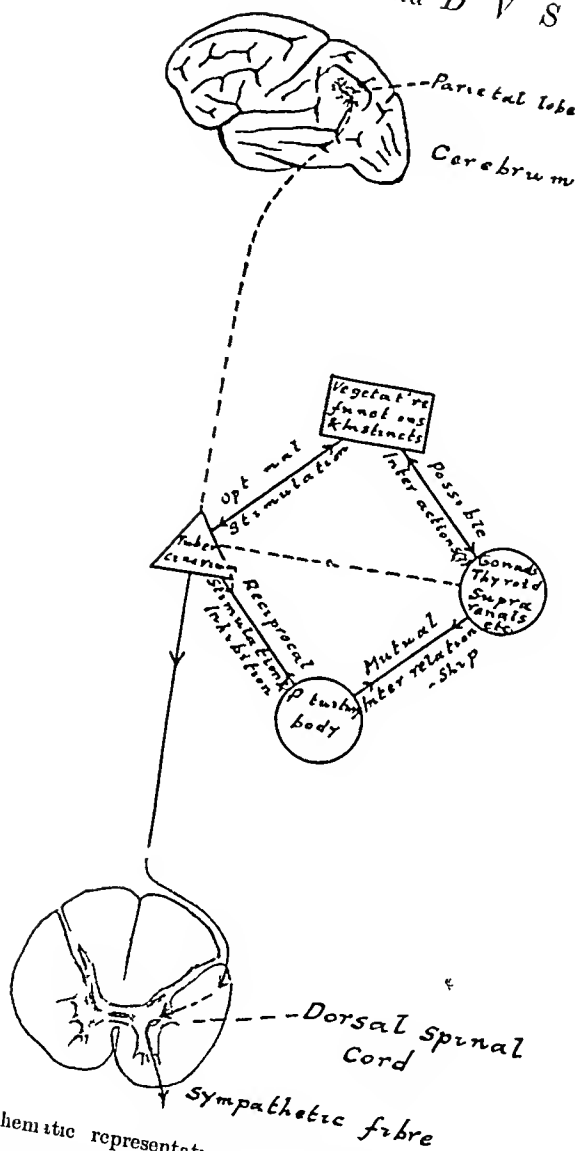
TEXT FIGURE 2 —Schematic representation of the inter relationships of the neuro hypophysial mechanism

The primitive tuberal nuclei which regulate the vegetative functions and instincts are stimulated by the hormones of the pituitary body carried by the hypophysio portal vessels   The liberation of an adequate amount of the hormones is assured by means of the reciprocal inter relationship between the pituitary body and the tuberal nuclei which are connected together by bundles of non medullated fibres   The activity of the pituitary body (which is ' the key stone of the endocrine arch ') is also influenced by other endocrine organs regulating the vegetative reactions   A possible inter relationship between the tuberal nuclei and the other endocrine organs is also suggested

The *tuber cinereum* is under the cortical control of certain regions of the parietal lobe and also sends many non medullated nerve fibres by way of the spinal cord to the thoracic sympathetic system The continuous lines indicate the nerve pathways and the inter relationships that are known to exist and the broken lines represent the possible connections that are likely to exist

(6) Evidence is also put forward to show that the hypothalamus which is regarded as the chief ganglion of the sympathetic system is to a certain extent under the control of the parietal lobe and is also connected by way of the spinal cord to the thoracic sympathetic system

(7) The neuro-hypophysial mechanism is very extensive and plays an important rôle in the normal vegetative functions of the organism, as shown diagrammatically in Text-fig 2

Key to lettering in figures —
B = water-bath
b c = blood corpuscles
C_1, C_2, C_3 = Clips attached to the rubber tubing
F = funnel
hp V_1 = hypophysio portal vessel
M = manometer
P b gr = Prussian blue granules
R = reservoir
S = sink
S a_1 = systemic vessel
T = water tap
t c = *tuber cinereum*
t_1, t_2, t_3 = stop cocks
V C = Venous cannula
W = Wolf's bottle
1, 2, 3 4 = ' L ' shaped tubes passing through the rubber stopper of the reservoir

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THE NON-GLUCOSE REDUCING BODIES IN BLOOD AND THEIR VARIATION IN SUGAR TOLERANCE TESTS, WITH SPECIAL REFERENCE TO CIRRHOSIS OF THE LIVER

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THIS investigation was originally intended to study the carbohydrate metabolism in detail in cases of cirrhosis of the liver We found that in most of our cases of cirrhosis of the liver the fasting blood sugar [estimated by the technique employed by Folin and Wu (1923)] was much below the lower limit of the normal (Radhakrishna Rao, 1934) With a view to see whether this reduction of the fasting blood sugar in cirrhosis affected the true glucose or the ' non-glucose reducing bodies ', which are also estimated with the glucose by the Folin-Wu method, a detailed investigation was carried out in cases of cirrhosis of the liver as well as in healthy controls Incidentally, we studied a few cases of diabetes mellitus as regards the non-glucose fraction of the apparent blood sugar The observation of Fontès and Thivolle (1929, 1930) that at least a portion of the non-glucose fraction, which they call ' glucid X ', varies with the free glucose after injection of adrenalin and insulin, induced us to study the variation of this fraction at regular intervals after glucose administration with the hope that any characteristic relationship between the two, which may be revealed by this study, may throw light on the nature of the ' non-glucose reducing bodies ' Though it is difficult to interpret the several observations recorded in this paper in the present state of our knowledge of the subject, still they are presented

with the hope that they might be useful to research workers pursuing this line of investigation

It is now generally agreed that the oxidization methods usually employed in the estimation of blood sugar give a higher figure than the amount of glucose actually present   This is due to the fact that in addition to the glucose there is also present in the blood another substance or substances capable of reducing the reagent used in the above methods   This non-glucose reducing substance or ' glucid X ' of Fontès and Thivolle (*loc cit*), which, according to Best (1919), is at least partly of the nature of a pentose, varies with the protein precipitant and the oxidizing agents used in the different methods for the estimation of blood sugar   Estimated as glucose by the Folin-Wu method (*loc cit*) it is on an average equivalent to 20 mg of glucose per 100 c c of blood   The different values obtained in the same sample of blood by the different methods for the estimation of blood sugar is mainly due to the variations in this non-glucose reducing body (glucid X), the true sugar remaining the same   ' Glucid X can be estimated as the difference between the values obtained in blood before and after fermentation with yeast as it has been shown that glucose in the blood is completely removed by treatment of the whole blood with an equal quantity of yeast suspension for about five minutes (Somogyi, 1927 1928 , Fontès and Thivolle, 1929, 1930)   But sometimes the ' glucid X ' is so modified by the yeast suspension that it may not be sensitive to some of the reagents used in the subsequent determination   Pickard, Pierce, Marsden, Tanaka and Townsend (1932) showed that ' glucid X ' is normal in nephritic bloods and that it is occasionally fermented by yeast   Pickard and Godwin (1932) pointed out that ' glucid X ' fermented with yeast completely in three days, and that occasionally it is destroyed with the glucose   Folin and Svedberg (1929) and Pickard and Godwin (*loc cit*) found that ' glucid X ' was normal in diabetes, while Somogyi and Krammer (1928) showed that it diminished in bad cases of diabetes where the high blood sugar persisted   Pickard *et al* (*loc cit*) suggest that it is a higher sugar on the basis of the work of Hubbard and Deegan (1928)

It will thus be seen that the value obtained by the Folin and Wu (*loc cit*) method for the estimation of blood sugar is the sum of the true glucose and the ' glucid X ' present in it

Pickard and his associates (*loc cit*) showed that there is another reduction called the ' y-reduction ' which is constantly present over that of glucose and ' glucid X ' when the blood sugar is estimated by the Benedict modification of the Lewis-Benedict method (quoted by Pickard *et al* , *loc cit*)   The ' y-reduction ' can also be estimated by ' subtracting the reduction by the Folin-Wu method (glucose plus glucid X) from the reduction, as glucose, in the same tungstate filtrate, by the Ionesco method (Pickard and Godwin, *loc cit*) '   Thus, the Benedict modification of the Lewis-Benedict method and the Ionesco (1930) method are sensitive to all the three reducing fractions, namely, the true glucose of the blood, the ' glucid X ', and the y-reduction   Pickard and his associates also showed that the substances causing y-reduction vary considerably in normal blood (from 14 mg to 68 mg with an average of 40 4 mg per cent) and that they are increased in the blood of nephritics Pickard and Godwin pointed out that ' the substances causing the y-reduction are increased over the normal in diabetic blood, but vary considerably '

According to Benedict and Newton (1928, 1929) glutathione of the blood is one of the chief non-glucose reducing substances and it raises the glucose content of the

blood by the Folin and Wu technique by about 10 mg to 20 mg per cent   Pickard (1932) showed that probably the glutathione of the cells is mainly responsible for the γ-reduction   Glutathione is found in practically all the tissues of the body, and in blood it is present in the red blood cells but not in the plasma   In a later communication Benedict (1931) stated that glutathione does not contribute to any appreciable extent to the saccharoid content of the blood   This is confirmed by Fashena (1933) who has shown that only 37 per cent of the saccharoid value of blood by the Folin-Wu method can be accounted for by glutathione   Somogyi (1927) mentions the possibility of thionene contributing to the reduction exerted by tungstic acid filtrates   But Benedict *et al* (1929) have shown that its total reducing power is so low that the quantity generally present in the blood cannot account for more than 1 mg or 2 mg of the apparent sugar content

TECHNIQUE

(*i*) The true glucose content of the blood was estimated by the copper precipitation method suggested by Somogyi (1931)   In this the proteins of the blood and the ' glucid X ' are precipitated by copper   The details are as follows —

One volume of blood is laked in seven volumes of distilled water and to it is added one volume of 7 per cent copper sulphate solution   After shaking the resulting solution one volume of 10 per cent sodium tungstate solution is added drop by drop   The solution is well shaken and filtered, using starch-free filter paper   The sugar content of the protein-free filtrate is estimated in the usual way by the Folin and Wu method

(*ii*) In the same sample of blood the protein-free filtrate is prepared in the usual way by using 10 per cent sodium tungstate and 2/3 N sulphuric acid solutions, and the sugar content of the resulting filtrate was estimated by the Folin-Wu method

The difference between the two methods (*i* and *ii*) gives the value of ' glucid X '

(*iii*) The y-reduction is obtained by subtracting the result obtained in (*ii*) from that obtained by the Ionesco method (quoted by Pickard and Godwin, 1932) described below —

To 5 c c of the tungstic acid filtrate in an Erlenmeyer flask are added 2 c c of normal sodium hydroxide solution, 1 c c of ferricyanide reagent* and 10 c c of distilled water   In another flask the same reagents are added to 5 c c of distilled water for control   They are kept in a boiling water-bath for 12 minutes and then cooled   If after cooling the solution is colourless, the procedure is again repeated with half the quantity of the protein-free filtrate   After cooling, 5 c c of 20 per cent sulphuric acid (iron-free) are added, and the resulting solutions are titrated with N/60 potassium permanganate solution using a microburette   The solution in the

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* *Ferricyanide reagent* —

Potassium ferricyanide	23 g
Potassium hydroxide	23 g
Distilled water	1,000 c c,

control flask is first titrated to a rose colour, and the reading noted   The contents of the other flask are then titrated to match the colour of the control   The second reading less that of the control gives the exact quantity of N/60 potassium permanganate required for titration   The number of c c of potassium permanganate solution used multiplied by 100 gives the amount of glucose in milligrams per 100 c c of blood

(iv) The glutathione of the blood was estimated by the iodine titration method described by Platt (1931)

Five c c of oxalated blood was laked in 10 c c distilled water, and to it was added 10 c c of 20 per cent trichloracetic acid   The resulting mixture was well shaken and filtered   Ten c c of the filtrate was titrated with freshly prepared N/1,000 iodine solution* from a microburette to a light-blue colour using soluble starch (2 drops of a 2 per cent soluble starch solution freshly prepared each week) as indicator   The number of c c of N/1,000 iodine solution used multiplied by 15 35 gives in milligrams the amount of glutathione contained in 100 c c of blood

According to this method the normal amount of glutathione present in the blood is about 30 mg to 50 mg per 100 c c (Platt *loc cit*)

(v) The inorganic phosphorus of the blood was estimated by a slight modification of Briggs' (1922) method   The total acid-soluble phosphorus was estimated by the same method after digestion of the trichloracetic acid filtrate with sulphuric acid using a minute amount of potassium persulphate to complete the digestion   The ester phosphorus is obtained as the difference between the acid-soluble phosphorus and the inorganic phosphorus (Kay and Byrom, 1927)

Our procedure in the present investigation was as follows   Samples of oxalated blood were taken before and at regular intervals (given in Tables IV to VIII) after the administration of glucose or lævulose by mouth   All the tests were done in the morning on hospital patients, who were kept starving overnight and received no breakfast   Patients who were admitted into the ophthalmic clinic of the King George Hospital, Vizagapatam, for eye complaints, and in whom the general condition was very good, formed the controls in this series   Pure glucose (Kahlbaum) and lævulose were used throughout, and the quantity given by mouth varied with the weight of the patient (1 g per kilo of body-weight)

In each sample of blood, as soon as possible after removal, the blood sugar by the different methods described above (Folin and Wu, Somogyi, and Ionesco) and the glutathione was estimated   In addition we also estimated the inorganic phosphorus, the total acid-soluble phosphorus, and the ester phosphorus in some of the cases with a view to study the possibility of any phosphoric acid compound of glucose contributing to the v-reduction   The urine of each patient was examined as a routine before and after the administration of sugar

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* N/10 iodine solution   2 c c
25 per cent potassium iodide solution   2 c c
Distilled water   200 c c

Results

The results obtained in some of the normal cases are given in Table I —

Table I

Showing the non-glucose reducing bodies in blood in some healthy controls (mg per 100 c c of blood)

Serial number	Case number	Glucid X	y reduction	Glutathione
1	3	18 8	69 1	49 12
2	11	12 8	196 6	33 00
3	12	9 2	89 9	22 26
4	16	10 6	103 3	24 56
5	17	11 1	114 7	30 32
6	18	8 3	108 8	37 22
7	31	6 1	104 8	36 07
8	Dr V K N	17 6	33 79	50 19
	,	18 2	48 93	
9	S₁4	12 8	56 7	
10	S₂21	18 6	50 4	
	Average	13 1	89 1	35 34

It will be seen that the non-glucose reducing bodies in blood show a great variation even in healthy people   The average value of glutathione in this series is within the normal limits given by Platt, while the ' glucid X ' and ' y-reduction ' are lower and higher respectively than the values given by Pickard and his associates

Tables II and III show the results obtained in some typical cases of cirrhosis of the liver and in a few cases of diabetes, nephritis, etc   Even here the non-glucose reducing bodies show a great variation   But, taking the normal standards obtained in this series (Table I), the average values of ' glucid X ' and ' y-reduction ' in cases of cirrhosis of the liver do not show much difference, while the glutathione content is lower than normal   In two cases of diabetes mellitus the ' glucid X ' shows a wide variation, but the ' y-reduction   though fairly constant, is much below the normal

TABLE II

Showing the non-glucose reducing bodies in blood in some typical cases of cirrhosis of the liver (mg per 100 c c of blood)

Serial number	Case number	Blood sugar (Folin-Wu)	Blood sugar (Somogyi copper filtrate)	'Glucid X'	Ionesco method	y reduction	Glutathione
1	1	85 2	48 4	36 8	176 2	91 0	
2	2	93 4	82 6	10 8	181 0	87 6	14 007
3	$M_1 5$	95 2	75 7	19 5	135 0	39 8	
4	6*	70 7	61 3	9 4	164 0	93 3	36 456
5	7	85 5	71 4	14 1	155 0	69 5	36 840
6	8	126 6	108 1	18 5	209 0	82 4	34 921
7	9	67 8	57 8	10 0	175 0	107 2	31 084
8	10	105 3	87 3	18 0	183 0	77 7	28 397
9	15	79 0	69 0	10 0	202 0	123 0	26 095
10	21	91 3	80 2	11 1	206 0	114 7	34 538
11	20	76 6	68 3	8 3	199 0	122 4	31 851
12	23	73 5	64 9	8 6	179 0	105 5	
13	24	81 6	74 6	7 0	197 0	115 4	19 955
14	$M_1 1$	69 7	51 0	18 7	117 17	47 47	
Average				14 0		91 1	29 41

* Clinical diagnosis was verified post-mortem in this case

standard   But, compared to the standard of Pickard and his associates these readings show an increase in the substances causing y-reduction   In one case of sprue the y-reduction is diminished, while in another case of ankylostomiasis the glutathione shows a lower reading than normal   The non-glucose reducing bodies do not show much difference from normal in one case of nephritis But the y-reduction in this case is certainly increased if the normal standard of Pickard and his associates is considered

TABLE III

Showing the non-glucose reducing bodies in blood in other cases
(mg per 100 c c of blood)

Serial number	Case number	Blood sugar (1 olm Wu)	Blood sugar (Solnogyi copper filtrate)	'Glucid X'	Ionesco method	y reduction	Glutathione	Disease
1	M₁	85 5	72 7	12 8	126 86	41 36		Sprue ?
2	M₂4	344 8	337 6	7 2	402 0	57 2		Diabetes mellitus
3	S₁42	304 0	269 2	34 8	372 0	68 0		Do
4	5	74 1	59 3	14 S	149 0	74 9	24 79	Ankylostomiasis— ascites
5	4	86 9	70 2	16 7	159 0	72 1	35 69	Subacute nephritis— ascites

The variations of the non-glucose reducing bodies in glucose and lævulose tolerance tests in normal people are given in Table IV   It will be seen that the 'glucid X' and glutathione show a rise, though to a slight extent only, similar to that of the blood sugar (Chart 1)

But, the y-reduction shows a considerable fall, most marked at the end of the first hour, with the rise in the blood sugar level   From the end of one hour it tends to rise again, but in some cases the fall is continued, and the y-reduction may actually become negative (Chart 2)   This fall in the y-reduction is a constant feature in all cases   In one case (Case No 17, Table IV) in which the non-glucose reducing bodies were estimated after the administration of lævulose, the same features described above are noticed

CHART 1
(Case No 17, Table IV)
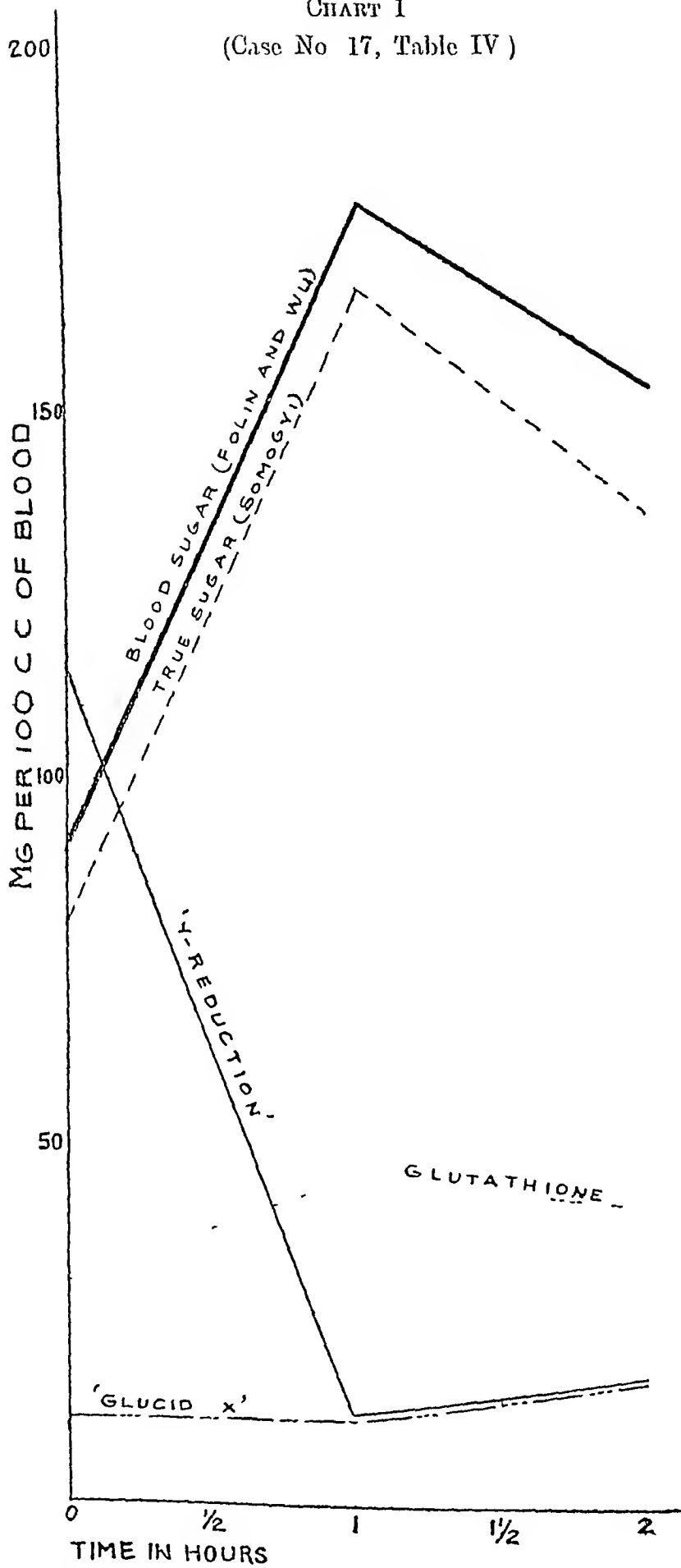
Showing the variations of non glucose reducing bodies in glucose tolerance test in a normal person

CHART 2
(Case No 12, Table IV)
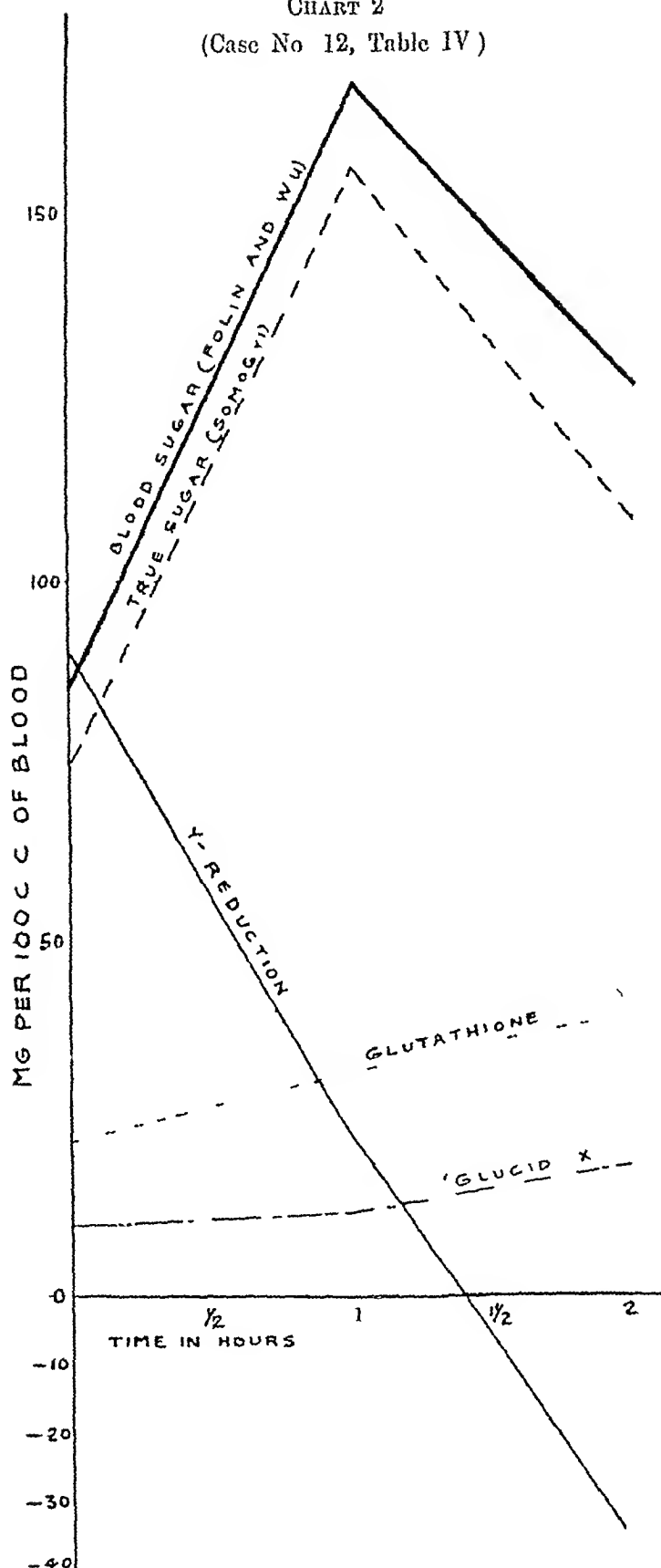
Showing the variations of non glucose reducing bodies in glucose tolerance test in a normal person In this case the y-reduction became negative at the end of two hours

TABLE IV

Showing the variations of the non-glucose reducing bodies in sugar tolerance tests in some healthy controls (mg per 100 c c of blood)

Serial number.	Case number	Time in hours after giving glucose (F= fasting)	Blood sugar (Folin Wu)	Blood sugar (Somogyi copper filtrate)	'Ghead X'	Ionesco method	y reduction	Glutathione
1	11	F	78 4	65 6	12 8	275 0	196 6	33 002
		1	108 1	95 2	12 9	167 0	58 9	32 618
		2	93 0	70 4	12 6	177 0	94 0	37 070
2	12	F	86 1	76 9	9 2	176 0	89 9	22 258
		1	168 0	156 2	11 8	191 0	23 0	31 468
		2	126 6	108 1	18 5	94 0	−32 6	39 143
3	16	F	84 7	74 1	10 6	188 0	103 3	24 560
		1	114 9	106 9	8 0	166 0	51 1	24 821
		2	131 6	125 0	6 6	183 0	51 4	24 560
4	17	F	91 3	80 2	11 1	206 0	114 7	30 316
		1	178 6	166 7	11 9	192 0	13 4	45 282
		2	152 7	135 1	17 6	171 0	18 3	41 061
5	18	F	95 2	86 9	8 3	204 0	108 8	37 224
		1	149 2	135 1	14 1	199 0	49 8	39 143
		2	117 6	103 1	14 5	163 0	45 4	32 035
6	31	F	80 2	74 1	6 1	185 0	104 8	36 073
		½	96 6	90 9	5 7	141 0	44 4	36 073
		1	98 0	90 9	7 1	143 0	45 0	38 375
		1½	101 0	94 6	6 4	148 0	47 0	36 073
		2	101 5	95 2	6 3	162 0	60 5	37 608
7	S₁4*	F	86 9	74 1	12 8	143 6	56 7	
		½	93 9	79 7	14 2	142 72	48 82	
		1	110 6	95 2	15 4	157 69	47 09	

* Levulose tolerance test

The behaviour of the non-glucose reducing bodies in sugar tolerance tests in cases of cirrhosis of the liver are given in Table V The same variations noticed in normal cases are also seen here (Charts 3 and 4) and the results do not much differ

from those seen in Table IV   In a case of cirrhosis of the liver (Serial No 11, Table V)
in which the non-glucose reducing bodies were estimated in lævulose tolerance test,
the 'glucid X' and y-reduction gave irregular results
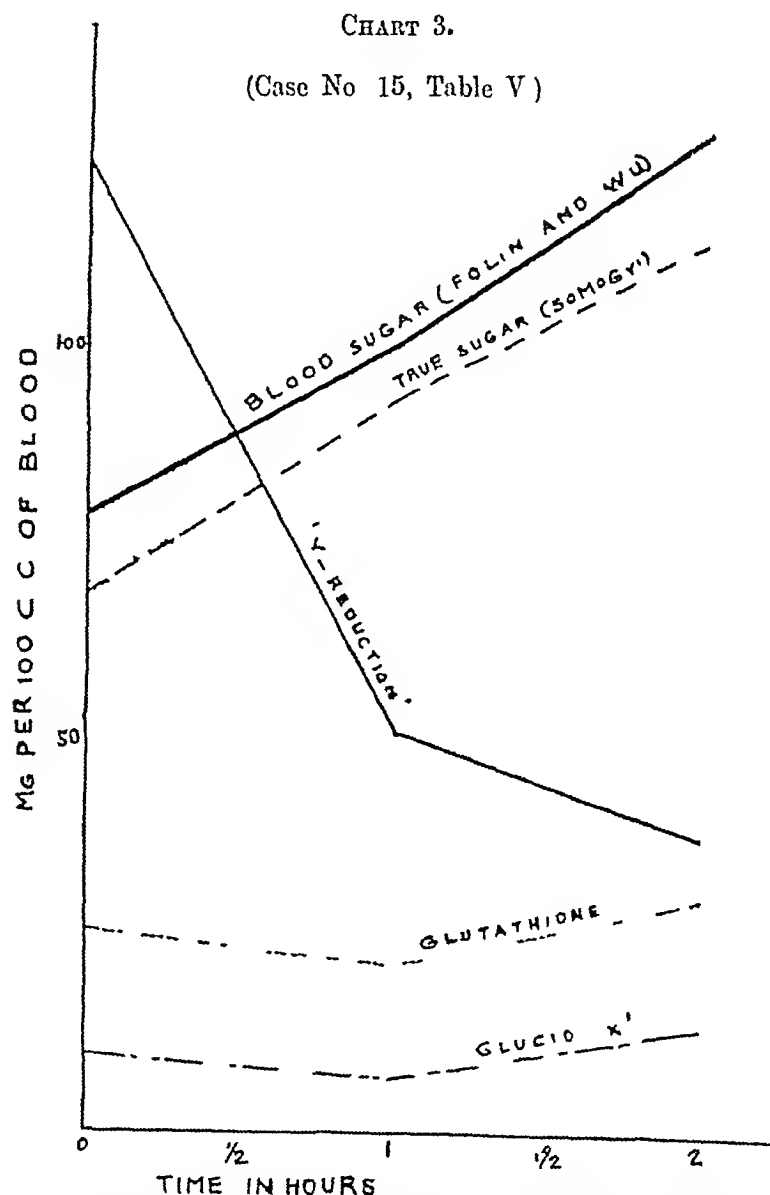
CHART 3.

(Case No 15, Table V)

Showing the variations of non-glucose reducing bodies in glucose tolerance
test in a case of cirrhosis of the liver

CHART 4
(Case No 2, Table V)
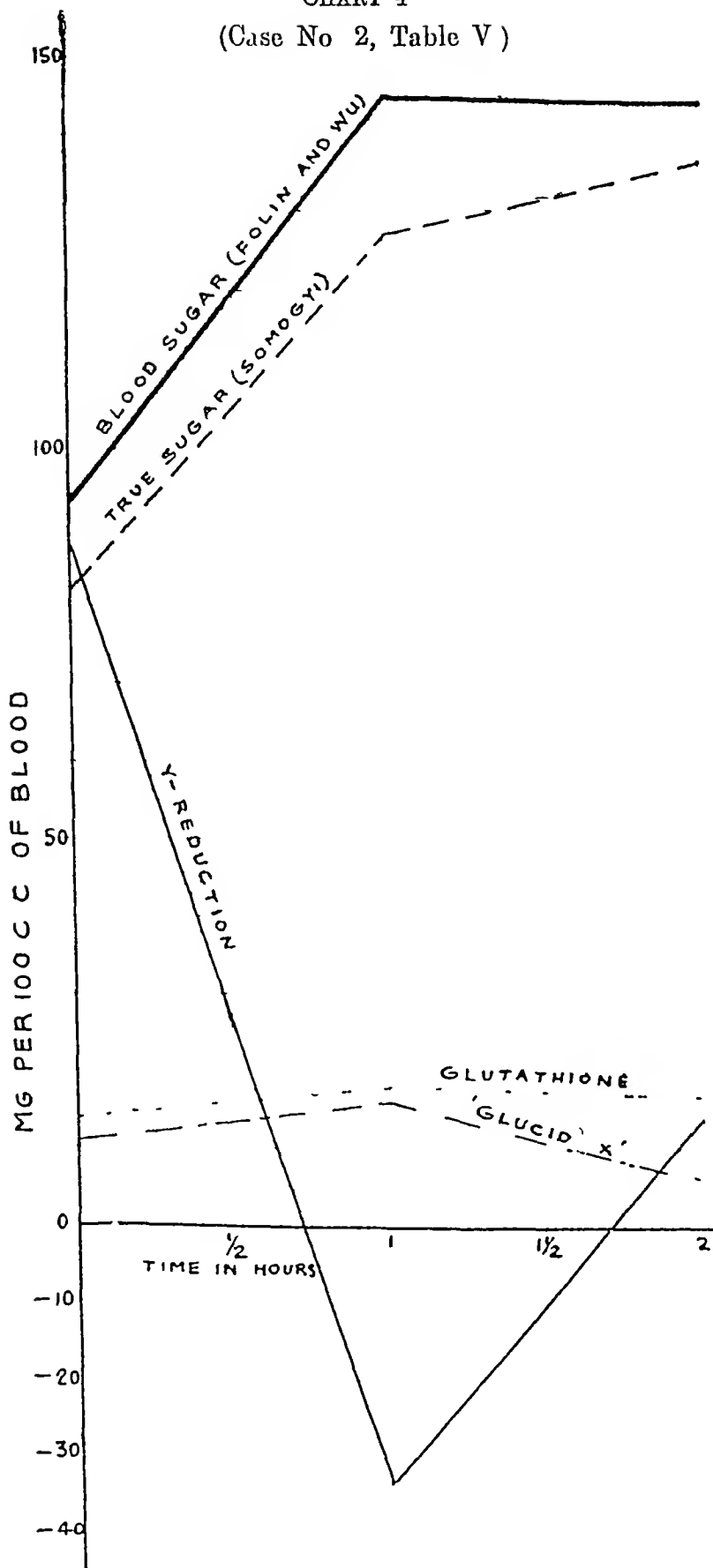
Showing the variations of the non glucose reducing bodies in glucose tolerance test in a case of cirrhosis of the liver. The y-reduction becomes negative in this case at the end of one hour. The macroscopic and microscopic appearances of the liver examined post mortem were those of toxic cirrhosis

TABLE V

Showing the variations of the non-glucose reducing bodies in sugar tolerance tests in some typical cases of cirrhosis of the liver
(mg per 100 c c of blood)

Serial number	Case number	Time in hours after giving glucose (I = fasting)	Blood sugar (Folin Wu)	Blood sugar (Somogyi copper filtrate)	'Glucid X'	Ionesco method	v reduction	Glutathione
1	2*	I	93 4	82 6	10 8	181 0	87 6	14 007
		1	143 9	127 4	16 5	111 0	−32 9	18 420
		2	142 8	136 0	6 8	157 0	14 2	17 139
2	7	F	85 5	71 4	14 1	155 0	69 5	36 840
		½	93 0	76 3	16 7	142 5	49 5	33 000
		1	125 0	102 0	23 0	130 0	5 0	36 070
		2	132 4	113 0	19 4	155 0	22 6	41 900
3	8	F	126 6	108 1	18 5	209 0	82 4	34 921
		1	215 0	186 9	28 1	223 0	8 0	33 386
		2	186 9	153 8	33 1	251 0	64 1	37 991
4	9	F	67 8	57 8	10 0	175 0	107 2	31 084
		1	97 6	86 2	11 4	131 0	33 4	32 926
		2	100 0	85 1	14 9	154 0	54 0	40 294
5	10	F	105 3	87 3	18 0	183 0	77 7	28 397
		1	250 0	224 7	25 3	242 0	−8 0	34 153
		2	161 3	134 2	27 1	185 0	23 7	36 840

* Clinical diagnosis was verified post mortem in this case

TABLE V—*concld*

Serial number	Case number.	Time in hours after giving glucose (F = fasting)	Blood sugar (Folin Wu)	Blood sugar (Somogyi copper filtrate)	'Ghoud X'	Ionesco method	y reduction	Glutathione
6	15	F	79 0	69 0	10 0	202 0	123 0	26 095
		1	100 0	93 4	6 6	151 0	51 0	21 490
		2	126 6	113 0	13 6	165 0	38 4	30 316
7	21	F	91 3	80 2	11 1	206 0	114 7	34 538
		1	168 0	161 3	6 7	209 0	41 0	36 456
		2	162 5	157 5	5 0	238 0	75 5	35 689
8	20	F	76 6	68 3	8 3	199 0	122 4	31 851
		1	136 0	126 6	9 4	181 0	45 0	32 619
		2	135 1	118 3	16 8	194 0	58 9	33 386
9	23	F	73 5	64 9	8 6	179 0	105 5	
		$\frac{1}{2}$	96 1	92 6	3 5	159 0	62 9	
		1	117 6	114 3	3 3	189 0	71 4	
		$1\frac{1}{2}$	120 5	119 0	1 5	200 0	79 5	
		2	131 6	126 6	5 0	209 0	77 4	
10	24	F	81 6	74 6	7 0	197 0	115 4	19 955
		1	112 4	110 5	1 9	176 0	63 6	23 025
		2	131 6	124 2	7 4	206 0	74 4	24 560
11	M,5*	F	95 2	75 7	19 5	135 0	39 8	
		$\frac{1}{2}$	113 0	103 1	9 9	154 0	41 0	
		1	127 4	112 4	15 0	160 0	32 6	
		$1\frac{1}{2}$	108 1	98 0	10 1	147 0	38 9	
		2	106 4	86 9	19 5	135 0	28 6	

* Lævulose tolerance test

The non-glucose reducing bodies showed the same variation as in normal cases, in a case of nephritis (Serial No 1, Table VI) and in one case of diabetes mellitus (Serial No 2, Table VI) In another case of diabetes mellitus (Serial No 3, Table VI) the y-reduction showed the normal variation though the values for 'glucid X' were very irregular

TABLE VI

Showing the variations of the non-glucose reducing bodies in sugar tolerance tests in other cases (mg per 100 c c of blood)

Serial number	Case number	Time in hours after giving glucose (1 = fasting)	Blood sugar (Folin Wu)	Blood sugar (Somogyi copper filtrate)	'Glucid X'	Ionesco method	y reduction	Glutathione	Disease
1	4	F	86 9	70 2	16 7	159 0	72 1	35 69	Subacute nephritis— ascites
		1	85 1	70 4	14 7	22 0	−63 1	40 29	
		2	74 1	60 1	14 0	212 0	137 9	40 06	
2	M₂4	F	344 8	337 6	7 2	402 0	57 2		Diabetes mellitus
		½	333 2	332 0	1 2	410 0	76 8		
		1	661 2	615 2	46 0	630 0	−31 2		
		1½	615 6	559 6	56 0	580 0	−35 6		
		2	533 2	519 2	14 0	550 0	16 8		
3	S₁42	F	304 0	269 2	34 8	372 0	68 0		Diabetes mellitus
		½	382 8	360 4	22 4	450 0	67 2		
		1	490 8	427 6	63 2	526 0	35 2		
		1½	476 0	459 6	16 4	518 0	42 0		
		2	442 4	412 4	30 0	500 0	57 6		

Tables VII and VIII are examples of the simultaneous variations in the phosphorus distribution in the blood after glucose administration   Our results seem to exclude the possibility of any (? unidentified) hexose-phosphate contributing to the y-reduction*

* Robison (1932) speaks of a small fraction of the acid soluble phosphoric ester in blood, ' possessing marked reducing properties and was thought to contain a *hexose phosphate* '

TABLE VII

Showing the phosphorus distribution and the non-glucose reducing bodies after glucose administration in a case of cirrhosis of the liver (mg per 100 c c of blood)

Time in hours after glucose (F=fasting)	Blood sugar (Folin-Wu)	Blood sugar (Somogyi copper filtrate)	'Glucid X'	Ionesco method	γ reduction	Glutathione	Inorganic phosphorus	Total acid soluble phosphorus	Ester phosphorus
F	81 6	74 6	7 0	197 0	115 4	19 96	2 325	17 310	14 985
1	112 4	110 5	1 9	176 0	63 6	23 03	2 247	20 550	18 303
2	131 6	124 2	7 4	206 0	74 4	24 56	2 181	18 300	16 119

TABLE VIII

Showing the phosphorus distribution and the non-glucose reducing bodies after glucose administration in a healthy person (mg per 100 c c of blood)

Time in hours after giving glucose (F=fasting)	Blood sugar (Folin-Wu)	Blood sugar (Somogyi copper filtrate)	'Glucid X'	Ionesco method	γ reduction	Glutathione	Inorganic phosphorus	Total acid soluble phosphorus	Ester phosphorus
F	80 2	74 1	6 1	185 0	104 8	36 07	3 279	20 820	17 541
½	96 6	90 9	5 7	141 0	44 4	36 07	3 159	19 980	16 821
1	98 0	90 9	7 1	143 0	45 0	38 38	2 988	21 210	18 222
1½	101 0	94 6	6 4	148 0	47 0	36 07	3 333	20 190	16 857
2	101 5	95 2	6 3	162 0	60 5	37 61	2 955	20 820	17 865

COMMENT

Our results indicate that the non-glucose reducing bodies vary within wide limits in health and disease   In spite of the fact that the Folin and Wu method

gives a higher reading than the amount of glucose actually present in the blood, the clinical interpretation of the results obtained by this method is usually correct as the reduction due to ' glucid X ' is generally small

The non-glucose reducing bodies in blood do not seem to be in any way responsible for the low fasting blood sugar seen in cases of cirrhosis of the liver, as the average value of ' glucid X ' and of y-reduction in cirrhosis cases and in normal persons is the same Moreover, though the glutathione content of the blood is lower than normal in cirrhosis cases, it cannot be considered to be responsible for the low blood sugar content found in the latter cases, as the ' glutathione accounts for little of the saccharoid content of the blood ' (Benedict, *loc cit*) Platt (*loc cit*) also obtained low readings for glutathione in cases of cirrhosis of the liver

In most of the sugar tolerance tests the variations in ' glucid X ' were similar to those in the blood sugar level Thus, these results are in favour of the observations of Fontes and Thivolle, who as mentioned already, showed that ' glucid X ' increased or decreased with the increase or decrease of the blood sugar level produced by the action of adrenalin or insulin respectively

While ' glucid X ' varies in the same direction as the changes in the blood sugar level in sugar tolerance tests in health and disease, the y-reduction behaves in a reverse manner The fall in the y-reduction at the end of one hour in most of the cases is fairly constant The interpretation of the behaviour of these two fractions of the non-glucose reducing bodies in sugar tolerance tests can only be possible after determining their exact nature

Pickard (*loc cit*) thought that the y-reduction is probably mainly due to the glutathione of the cells Rawson and Pickard (1933) state that the non-glucose reduction is ' due to unknown substances, the amount of which increases in pathological blood, and to glutathione which represents approximately half of the non-glucose reduction in normal blood ' But the slight changes in the glutathione content of the blood compared to the enormous variations in the y-reduction in our sugar tolerance tests, in health and disease, indicate that glutathione cannot be considered to be mainly responsible for the y-reduction In fact, as the reducing power of glutathione is only 0 2 of its weight of glucose (Benedict, *loc cit*), in our series of healthy persons glutathione represents only 7 8 per cent of the y-reduction, although it represents 53 8 per cent of the ' glucid X ' In a few cases (Tables VII and VIII , Case 7, Table V), however, the amount of ' glucid X ' compared with the glucose equivalent of glutathione is so small as even to throw doubt on the rôle of the latter in contributing to the ' glucid X ' value Pickard and his associates (*loc cit*) showed that the y-reduction ' does not seem to have any relation to creatinine ' and consider that it may be due to several bodies Hubbard and Deegan (*loc cit*) and Bigwood and Wuillot (1929) also point out that neither uric acid nor creatinine contributes to the ' residual reduction '

Recently, our attention was drawn to a communication by van Eekelen, Emmerie, Josephy and Wolff (1933) on the presence of vitamin C in blood These authors are of opinion that it is ' very probable that the reducing factor in blood (and urine) is indeed vitamin C ' Since then we have been collecting material with a view to studying the relationship between the non-glucose reducing substances presented in this paper and vitamin C or ascorbic acid

SUMMARY AND CONCLUSIONS

The non-glucose reducing bodies in blood in health and disease, with special reference to cirrhosis of the liver, were studied, and their variations in sugar tolerance tests determined   It is pointed out that —

1   The non-glucose reducing bodies vary within wide limits in health and disease

2   The average values for ' glucid X ' and ' γ-reduction ' in cases of cirrhosis of the liver do not show much variation compared to the normal standards obtained in this series   The glutathione content of the blood, however, is diminished in these cases of cirrhosis of the liver

3   In most of the sugar tolerance tests in health and disease, ' glucid X ' and glutathione vary in the same direction as the blood sugar, while the γ-reduction shows a considerable fall (most marked at the end of one hour) with the rise in the blood sugar   In some cases the γ-reduction actually becomes negative

4   From the data at present available the non-glucose reducing bodies in blood do not seem to be in any way responsible for the low fasting blood sugar found in cases of cirrhosis of the liver

5   The glutathione of the blood cannot be considered to be mainly responsible for the ' γ-reduction '

6   A study of the ascorbic acid (vitamin C) content of the blood and its variations in sugar tolerance tests in these cases may throw further light on the nature of the ' non-glucose reducing bodies ' present in blood

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USE OF ALKALI TARTRATES IN DIABETES AND PROLONGED FASTING

BY

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IN several publications (*Jour Phys Chem*, 1925, **29,** p 926, 1928, **32,** p 1263, 1930, **34,** pp 711, 993, 1932, **36,** p 2504, Z *anorg Chem*, 1930, **191,** p 150) we have shown that in presence of inductors like ferrous hydroxide, cerous hydroxide, manganous hydroxide, glutathione, insulin, etc, which can take up oxygen directly from air under ordinary conditions, solutions or suspensions of food materials like starch, sugars, proteins, egg-yellow, egg-white, milk, amino-acids, etc, fats (butter, lecithin), glycogen and glycerol can be readily oxidized by passing air Moreover, it has been shown that solutions or suspensions of these food materials can be oxidized to carbon dioxide and water by simply passing air in presence of sunlight In presence of sunlight, the induced oxidization of food materials is greatly increased

Experiments carried on in presence of light show that the amount of tartrate oxidized (15 6 per cent in sunlight) is greater than in the diffused light (3 per cent) of the laboratory Similarly other materials, such as glucose, starch, butter, egg-white and egg-yellow, glycogen, milk and lecithin, have been oxidized by passing air through solutions or suspensions of the above substances in presence of sunlight

It is known that visible light, especially rays of longer wave lengths, can penetrate the epidermis to appreciable depths and hence it has been suggested that light acts as a preventive and curative agent in diseases by acting not only as a stimulant of the body cells but also as an accelerator in the metabolism of food materials (*cf* DHAR, 1932, 'New Conceptions in Biochemistry')

Recently, we have been able to oxidize solutions of alkali tartrates to carbon dioxide and water by passing air through their solutions containing ferrous or cerous hydroxide Our experimental results show that, by the addition of sodium tartrate, the amount of potassium oleate oxidized in 30 hours by 73 litres of air is considerably decreased In the case of ferrous hydroxide as inductor, in presence of sodium tartrate the percentage of potassium oleate oxidized is 7 9, whilst under

identical conditions and in absence of sodium tartrate the percentage of oleate oxidized is 40 7   The corresponding results with cerous hydroxide as inductor are 16 8 per cent and 45 1 per cent respectively   Hence, it is clear that the addition of a tartrate markedly decreases the velocity of the oxidization of potassium oleate

These results seem important from the point of view of treatment of diabetes and prolonged fasting   It is established beyond doubt that the excretion of acetone bodies in the urine occurs only in conditions in which a carbohydrate is either not being utilized owing to metabolic deficiency or is missing from the food   To the former category belong the diseases like diabetes or cases of poisoning with drugs like phlorhizin , to the latter cases of continued vomiting or voluntary abstention from carbohydrate food   In many cases when acetonuria has made itself manifest as a result of lack of carbohydrate material in the food, the administration of such materials causes the prompt disappearance of ketosis   The authors have suggested the following explanation of the anti-ketogenic properties of carbohydrates   Under normal conditions, the heat and energy of the body are derived from the simultaneous combustion of carbohydrates proteins and fats   When, as a result of special conditions, carbohydrate is not burning, more fat and protein must be burnt in order to maintain the body temperature and activity at its normal level   Hence, in unit time, more fat would be burning than under normal conditions, and the combustion of fat would be incomplete, so that acetone bodies would be formed   It has been observed that whenever there is rapid combustion of fat, as for instance in hyper thyroidism, there is elimination of acetone bodies   The same is the case *in vitro* too   Chakravarti and Dhar (*Jour Ind Chem Soc* , 1929, **6**, p 617) have observed that, in the rapid oxidization of fatty acids by hydrogen peroxide and ferric salts, there is the formation of acetone bodies   They have also observed that if the rate of oxidization of fats be slowed down, as is the case when carbohydrates (e g , glucose) are present, the amount of acetone bodies formed diminishes   Palit and Dhar (*Jour Phys Chem* , 1930, **34**, p 710) further find that in the slow and induced oxidization of fats, acetone bodies are not formed at all, but only carbon dioxide and water, the products of normal metabolism

The experimental results obtained by us show that just as carbohydrates decrease the velocity of oxidization of the fats, similarly tartrates can also decrease the velocity of the oxidization of fat   Hence, a tartrate or a citrate, apart from its power of acting as a buffer in removing the excess of hydrogen ion H^o generated in the body, serves as an anti-ketogenic substance like a carbohydrate   Moreover, mild alkalis have been found to be helpful in increasing the oxidization of food materials   Consequently, in the treatment of nausea and other symptoms arising from prolonged starvation, or in diabetes, a mixture of sodium bicarbonate and an alkali tartrate or citrate is likely to be more efficacious than the usual practice of giving sodium bicarbonate alone, because the tartrate or citrate acting as a food and supplier of energy serves as a marked anti-ketogenic agent by decreasing the velocity of oxidization of fatty food materials and the formation of the acetone bodies   It seems pretty certain that when fats are quickly and incompletely burnt in the animal system, acetone bodies are formed and the real treatment in fasting and diabetes is the prevention or decrease of the formation of acetone bodies by causing slow but complete oxidization of the fats   Any agency, which will retard the velocity of the oxidization of fats in the body, will be helpful in the treatment of nauseating and other symptoms in fasting and in diabetes and such agencies are carbohydrates or

alkali tartrates or citrates   In acute diabetes, the animal system is almost unable to oxidize the carbohydrates and hence the system has to depend mainly on fat for its sustenance   Consequently, the diabetic has to burn more fat in an unit time than the non-diabetic and this leads to the formation of acetone bodies   If a soluble citrate or a tartrate or a mixture of both is given to the diabetic along with sodium bicarbonate, the diabetic is likely to utilize the citrate or tartrate as food and this will check the formation of acetone bodies by decreasing the velocity of oxidization of the fats   In long and continuous starvation also more or less the same thing happens   After the first days of starvation, the glycogen is exhausted and the body has to depend mainly on fats which, in the absence of carbohydrates, are rapidly and incompletely metabolized with the formation of acetone bodies   This is likely to be prevented or considerably decreased by the addition of citrates or tartrates along with sodium bicarbonate   The authors are of opinion that the citrates or tartrates in the drinks will prove highly beneficial in the treatment of long fasts, because in such cases the body has not lost the power of oxidization and will utilize the tartrates and citrates as food along with the fats of the body

Our experimental results show that, in presence of traces of copper, the induced oxidization of tartrate is markedly facilitated, whilst in larger concentrations it retards the same process   Hence, it appears that in the treatment of metabolism diseases or anæmia, the addition of traces of copper salts to iron or manganese may be useful

identical conditions and in absence of sodium tartrate the percentage of oleate oxidized is 40 7   The corresponding results with cerous hydroxide as inductor are 16 8 per cent and 45 1 per cent respectively   Hence, it is clear that the addition of a tartrate markedly decreases the velocity of the oxidization of potassium oleate

These results seem important from the point of view of treatment of diabetes and prolonged fasting   It is established beyond doubt that the excretion of acetone bodies in the urine occurs only in conditions in which a carbohydrate is either not being utilized owing to metabolic deficiency or is missing from the food   To the former category belong the diseases like diabetes or cases of poisoning with drugs like phlorhizin , to the latter cases of continued vomiting or voluntary abstention from carbohydrate food   In many cases when acetonuria has made itself manifest as a result of lack of carbohydrate material in the food, the administration of such materials causes the prompt disappearance of ketosis   The authors have suggested the following explanation of the anti-ketogenic properties of carbohydrates   Under normal conditions, the heat and energy of the body are derived from the simultaneous combustion of carbohydrates proteins and fats   When, as a result of special condi tions carbohydrate is not burning more fat and protein must be burnt in order to maintain the body temperature and activity at its normal level   Hence, in unit time, more fat would be burning than under normal conditions, and the combustion of fat would be incomplete, so that acetone bodies would be formed   It has been observed that whenever there is rapid combustion of fat, as for instance in hyper thyroidism, there is elimination of acetone bodies   The same is the case *in vitro* too   Chakravarti and Dhar (*Jour Ind Chem Soc* , 1929, **6,** p 617) have observed that, in the rapid oxidization of fatty acids by hydrogen peroxide and ferric salts, there is the formation of acetone bodies   They have also observed that if the rate of oxidization of fats be slowed down as is the case when carbohydrates (e g glucose) are present the amount of acetone bodies formed diminishes   Palit and Dhar (*Jour Phys Chem* , 1930, **34,** p 710) further find that in the slow and induced oxidization of fats, acetone bodies are not formed at all, but only carbon dioxide and water, the products of normal metabolism

The experimental results obtained by us show that just as carbohydrates decrease the velocity of oxidization of the fats, similarly tartrates can also decrease the velocity of the oxidization of fat   Hence, a tartrate or a citrate, apart from its power of acting as a buffer in removing the excess of hydrogen ion H^o generated in the body, serves as an anti-ketogenic substance like a carbohydrate   Moreover, mild alkalis have been found to be helpful in increasing the oxidization of food materials   Consequently, in the treatment of nausea and other symptoms arising from prolonged starvation, or in diabetes, a mixture of sodium bicarbonate and an alkali tartrate or citrate is likely to be more efficacious than the usual practice of giving sodium bicarbonate alone, because the tartrate or citrate acting as a food and supplier of energy serves as a marked anti-ketogenic agent by decreasing the velocity of oxidization of fatty food materials and the formation of the acetone bodies   It seems pretty certain that when fats are quickly and incompletely burnt in the animal system, acetone bodies are formed and the real treatment in fasting and diabetes is the prevention or decrease of the formation of acetone bodies by causing slow but complete oxidization of the fats   Any agency, which will retard the velocity of the oxidization of fats in the body, will be helpful in the treatment of nauseating and other symptoms in fasting and in diabetes and such agencies are carbohydrates or

EFFECT ON THYROID CELLS *IN VITRO* OF THE 'GOITRE-NOXA' ASSOCIATED WITH CERTAIN CONDITIONS OF INSANITATION

BY

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In previous papers (McCarrison and Madhava, 1932, McCarrison, 1933, McCarrison, 1933a) evidence was brought forward indicating the existence, under certain conditions of insanitation to which albino rats were subjected, of a 'goitre-noxa' of unknown nature It was shown that this noxa is water-soluble and possibly, therefore, water-borne

A solution of the noxa was prepared as follows 200 grammes of the compost contained in the insanitary cages in which the rats were confined (McCarrison and Madhava, 1932), were extracted with one litre of distilled water The mixture was allowed to stand in stoppered bottles for 48 hours or more, being vigorously shaken from time to time Thereafter, it was filtered through microbe-proof porcelain candles (Cheavin's) under pressure The filtrate so obtained is the solution of the 'noxa' with whose effect on cultures of thyroid cells *in vitro* this paper deals If ' n ' be the amount of ' noxa ' contained in each gramme of compost then the solution represents a 1 in 5 dilution of ' n ' The compost from which it was prepared consisted of a fermenting mass of rat excreta (fæces and urine) and vegetable matter (cabbage, whole-wheat, cholam), the latter being remnants of food that were allowed to accumulate in the cage Yeast had at one time been added to the compost and its activities were concerned in the fermentation processes going on therein To facilitate these processes the compost was kept moist

Material.

A 10 per cent solution of the ' noxa ' was made up in Tyrode solution   1 volume of the original solution in distilled water to 9 volumes of Tyrode solution , representing a 1 in 50 dilution of ' n '   This solution was passed through a sterilized Seitz filter under positive pressure and received into sterile test-tubes   These were plugged with sterile cotton-wool and kept in cold storage   Further dilutions in Tyrode solution were made from this solution as required

Method.

The hanging drop method of culture was employed , the thyroid being obtained from 18- to 20-day chick embryos (McCarrison and Sankaran, 1933)   *Control cultures* were made in equal parts of hen plasma and Tyrode solution , the *experimental cultures* were made in equal parts of hen plasma and ' noxa ' in various dilutions in Tyrode solution   No embryo extract was added to the cultures, complicating factors, which might have been introduced by the growth-promoting substances contained in this extract, being thus avoided   In each experiment six or more control cultures were put up side by side with six or more experimental cultures Care was taken always to use the same plasma in each series, for the growth of thyroid cells varies with the age of the plasma   the longer the period that has elapsed since its removal from the body the poorer the growth of the cells   The most striking results were obtained with 15-day old plasma whose growth-promoting properties were depreciated by age   Then, the stimulating effect of a suitable concentration of the noxa appeared in striking contrast to the control culture to which no noxa had been added   Plates II and III are representative of cultures in which 12-day old plasma was used

Experiments.

A preliminary series of control and experimental cultures showed that a 1 in 500 dilution of the ' noxa', representing a 1 in 1,000 concentration of ' n ' in the culture medium, completely inhibited thyroid growth

Further dilutions, representing concentrations of 1 in 10,000, 1 in 50,000, 1 in 250,000 and 1 in 500,000 of noxa in the culture medium, were made and cultures put up at each degree of concentration   It was found that the higher the concentration of noxa in the culture medium the greater the inhibition of growth At moderate degrees of concentration, such, for instance, as one of 1 in 250,000, growth occurred but degenerative changes set in early   It was observed, further, that the greater the dilution of the noxa, or the lower its concentration in the culture medium, the better the growth of the thyroid cells , best growth being obtained when the concentration was 1 in 500,000   At this degree of concentration the growth of the thyroid cells was markedly stimulated, and degenerative changes occurred no more rapidly than in controls   Quantitative estimates (*see* McCarrison and Sankaran 1933) of this stimulation were made   the results of these are set out in Tables I and II and are exhibited graphically in Figs 1 and 2

PLATE II
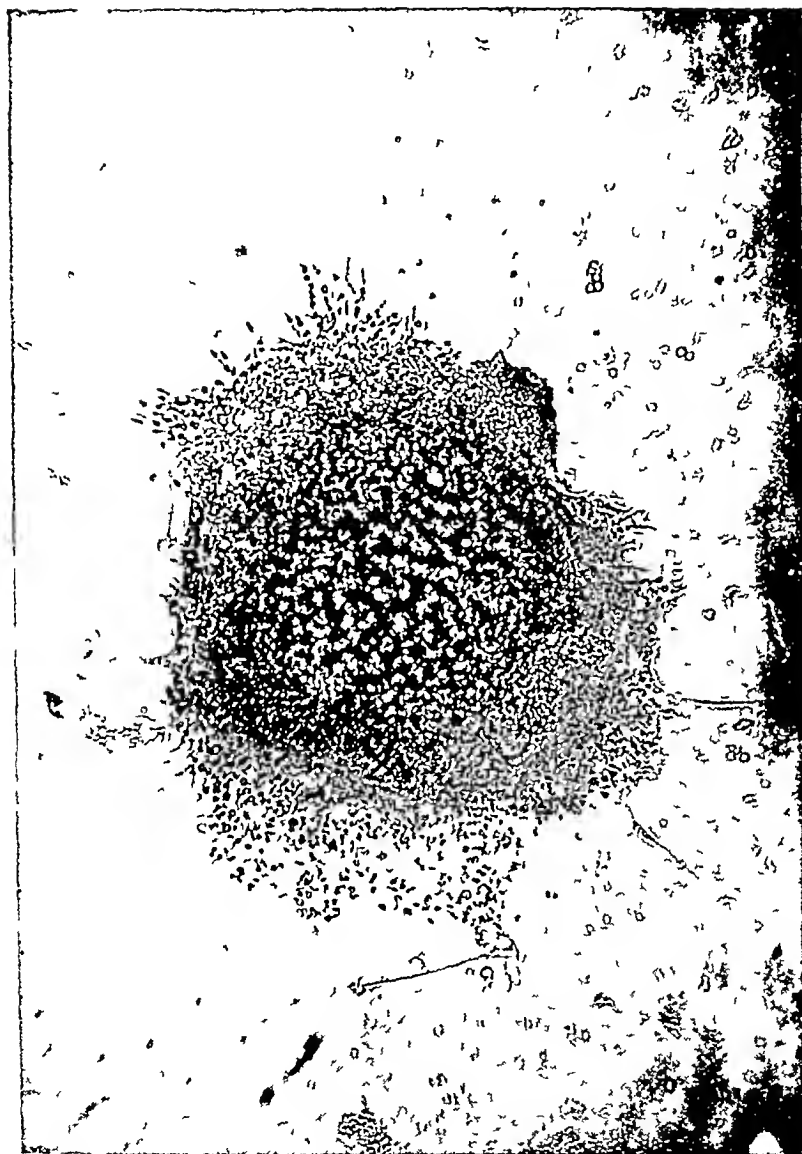
48 hour growth of 18 day old embryo chick thyroid in equal parts of 12 day
old fowl plasma and Tyrode solution
  Living, unstained, preparation
  Culture put up 21st March, 1933
  Compare with Plate III

PLATE III
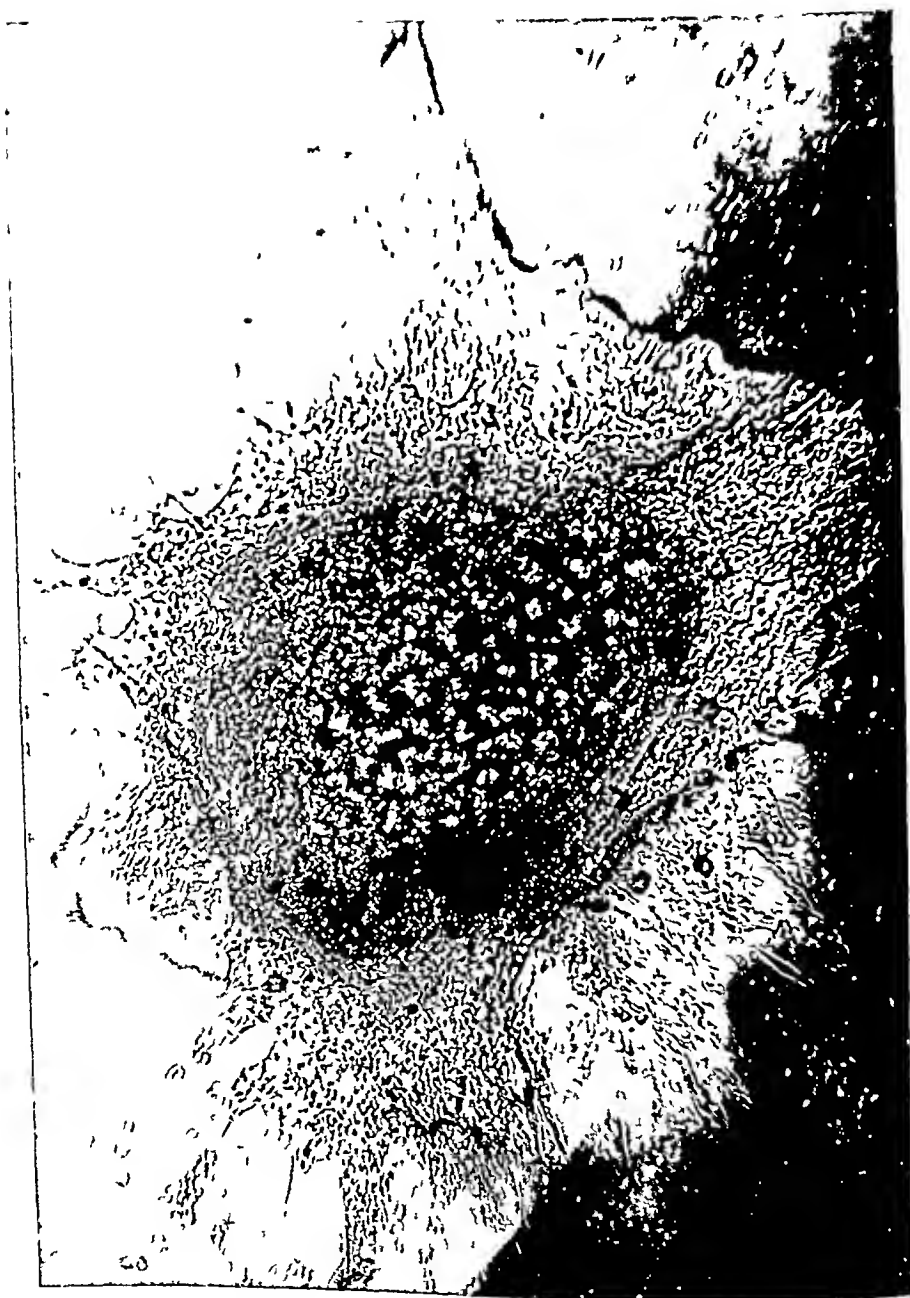
48 hour growth of 18 day old   embryo chick thyroid in equal parts of 12 day
old fowl plasma and Tyrode solution containing the noxa in a concentration
of 1 in 500,000   Same thyroid as in Plate II
    Living unstained, preparation
    Culture put up 21st March, 1933
    Compare with Plate II and note marked stimulation of growth

TABLE I

Showing the rate of growth in control cultures and in experimental cultures to which the goitre-noxa was added in a concentration of 1 in 500,000

	22nd March, 1933			23rd March, 1933			24th March, 1933	Area of original explant
Control cultures								
Slide No	11 a m 23 hrs	4 p m 28 hrs	10 p m 31 hrs	10 a m 46 hrs	4 p m 52 hrs	10 p m 58 hrs	10 a m 70 hrs	
1	3	15	42	88	131	180	Cystic	144
2	2	26	58	115	163	213	Cystic	120
3	4	16	38	66	96	Cystic		152
4	3	17	52	200	315	Cystic		160
5	14	48	96	172	268	502	Cystic	160
TOTALS	26	122	286	641	873	913		736
Total for 5 cultures	26	122	286	641	973	1 520		
Relative increase *	0 035	0 166	0 39	0 87	1 32	2 06		

TABLE II

Experimental cultures (Noxa 1 in 500,000)								
1	26	48	87	208	349	516	Cystic	164
2	12	83	157	303	574	818	Cystic	114
3	9	57	116	215	393	673	Cystic	184
4	6	30	99	195	370	Cystic		96
5	4	33	82	302	490	674	Cystic	108
6	6	43	81	146	248	456	Cystic	200
TOTALS	63	294	622	1,369	2 421	3,137		866
Total for 5 cultures	52	245	519	1,141	2,020	3 137		722
Relative increase *	0 072	0 34	0 72	1 59	2 84	4 35		

* Calculated from the formula $\dfrac{O + G}{O}$, where O is the area of the original explant and G the total area of growth
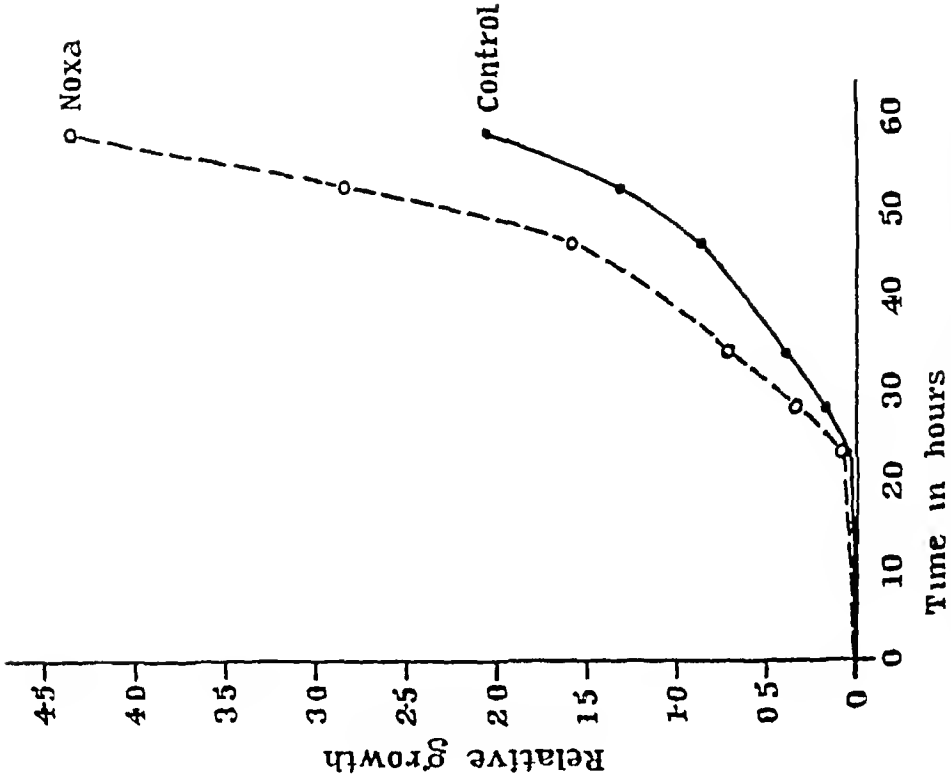
Fig 2 Relative growth curve of thyroid cells under the influence of the noxa (1 in 500,000) as compared with controls
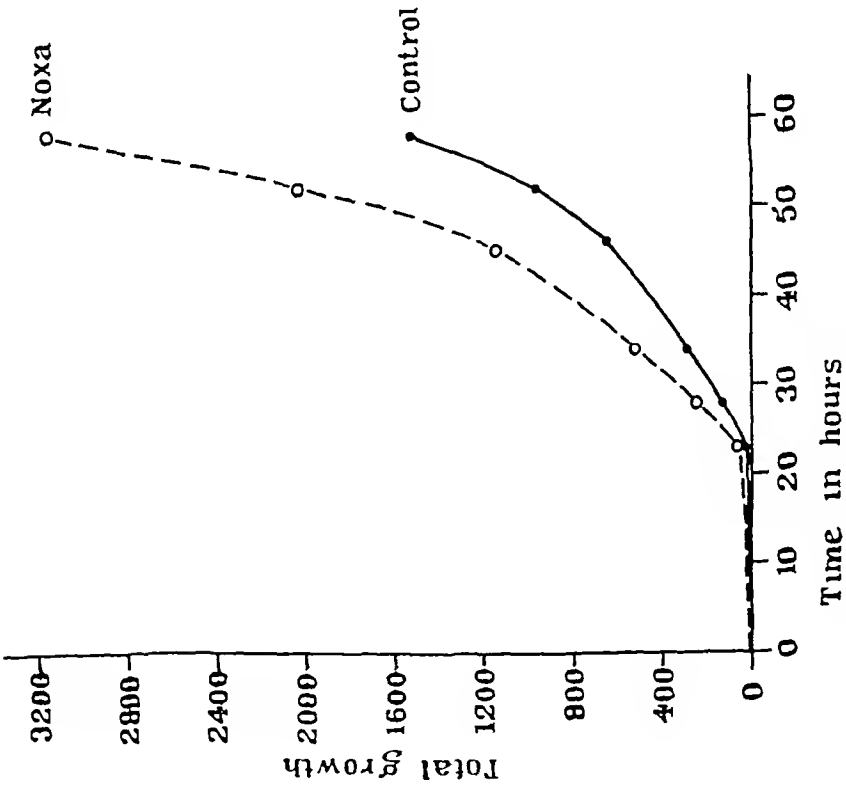
Fig 1 Actual growth curve of thyroid cells under the influence of the noxa (1 in 500,000) as compared with controls

EFFECT OF CAGING ON THE THYROID GLAND OF ALBINO RATS

BY

SIR ROBERT McCARRISON,

AND

B G KRISHNAN,

WITH A STATISTICAL NOTE

BY

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Two types of rat-cage are used in these Laboratories 'the triple cage' (Plate IV, fig 1) and 'the metabolism cage' (Plate IV, fig 2) In the course of our work on factors influencing the size of the thyroid gland certain anomalous results were observed in rats confined in the latter type of cage It was thought necessary, therefore, to examine the possibility that the type of cage might, *per se,* have an influence on the size of the thyroid gland of rats inhabiting these cages

The triple cage consists of a teak-wood frame ($36'' \times 12'' \times 12''$) lined on the inside with thin zinc sheeting, and enclosed, as to its sides and floor, by half-inch wire-netting It is divided into three compartments by two movable zinc partitions Each compartment measures $12'' \times 12'' \times 10.2''$ and is provided with a zinc lid and a removable zinc floor The latter is in position when the animal is feeding, thereafter it is removed, leaving the occupant on the netted wire floor through which the excreta drop to a tray below Each compartment is occupied by a single rat This type of cage, which is easily kept clean, admits of the animals confined in it taking free exercise, this they do by clambering about its netted wire walls

The metabolism cage consists of a cylinder ($9.1'' \times 7.25''$) of fine wire gauze (0.1 inch mesh), fitted into a flanged, and paraffin-lined, tin funnel, the mouth of the funnel being closed by a movable and paraffin-coated circular disc of wire

gauze (0 2 inch mesh) on which the rat rests   The top end of the cylinder is closed by a tin lid   A small recess is fitted to the side of the cylinder for the accommodation of the drinking water tube   This type of cage is difficult to keep clean, it does not admit of the occupant taking exercise

First Experiment.

Two groups of 48 young rats, identical as to age, sex-distribution and body weight, were taken from the same non-goitrous stock   One group was confined in triple cages, a single animal in each compartment   The other was confined in metabolism cages, a single animal in each cage   Both groups were fed alike on a modified 'stock diet'* consisting of whole-wheat flour *chapattis* lightly smeared with butter, sprouted gram (*legume*), fresh cabbage, fresh carrots and tap-water Whole milk was supplied in the proportion of 5 c c per rat per day   To ensure the equal distribution and consumption of the milk the total daily ration of *chapatti* for each group was soaked in the total daily ration of milk, until the latter was completely absorbed by the former   Test measurements revealed that the daily average food consumption was approximately the same in the two groups   The experiment was carried out during the spring and early summer of 1933 and was continued for 65 days   During its course one animal in the second group (metabolism cages) died   On the conclusion of the experiment the animals were weighed, killed and their thyroid glands removed   The thyroids were weighed with the usual precautions against loss of weight by drying

Results of the Experiment.

These are shown in the following table —

Number of rats (1)	Type of cage (2)	Initial average body weight g (3)	Average final body weight, g (4)	Average thyroid weight, mg (5)	Average thyroid size ('r') mg † (6)	Percentage incidence of true goitre ‡ (7)
48	Triple	60	125	13 9	11 0	*Nil*
47	Metabolism	60	126	22 1	17 5	72 3

† Weight in milligrams per 100 grammes of body weight

‡ Glands exceeding the normal mean size ('1') by more than 2 5 times the standard deviation from that mean (McCarrison and Madhava 1932)

Repeat Experiment

The details of this experiment were the same as in the previous one except that fewer rats were used, the animals were older and the experiments were continued for a longer period of time (186 days)   Four groups of rats were used, two of

---

*This diet was estimated to contain 21 γ of iodine per kilogram   The drinking water contained 0 45 γ of iodine per litre   *See* also foot note on page 61

which were fed on the same diet as in the first experiment and two on a diet consisting of 20 parts of whole-wheat flour, 20 parts of *cholam* (a kind of maize) and 60 parts of fresh raw cabbage * One group on each diet was confined in triple cages the other in metabolism cages

Results of Repeat Experiment

These are shown in the following table —

Number of rats	Type of cage	Diet	Initial average body weight g	Final average body weight g	Average thyroid weight mg	Average thyroid size (r) m_c	Percentage incidence of true goitre	Average iodine content (γ) of glands per cent fresh weight
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
10	Triple	Modified stock	89	139	17 3	12 9	*Nil*	0 0130
11	Metabolism	do	90	142	25 3	18 0	63 6	0 0050
10	Triple	C w c†	87	137	18 3	13 4	30 0	0 0154
12	Metabolism	do	88	146	29 9	20 9	91 6	0 0060

† C w c is an abbreviation for ' Cabbage wheat cholam '

Statistical Note.

The statistical constants of the thyroid-size (' r ', or the thyroid-weight in milligrams per 100 grammes of body-weight), determined on the theory that the groups examined were ' small samples ', are as follows —

	FIRST EXPERIMENT		REPEAT EXPERIMENT	
	Triple cages	Metabolism cages	Triple cages	Metabolism cages
Number of rats	48	47	20	23
Mean with standard error	11 04 ± 1 13	17 50 ± 0 58	13 17 ± 0 40	19 51 ± 1 03
Standard deviation	2 47	4 00	1 80	4 94
Co efficient of variability (per cent)	22	23	14	25

* This diet was estimated to contain on the average about 22 γ of iodine per kilogram But owing to the varying iodine content of cabbage at different seasons of the year in this locality, the figure is only approximate Thus, the inner leaves of ' winter ' cabbage contained 9 γ of iodine per kilo, the outer leaves, 14 γ The inner leaves of ' summer ' cabbage contain 9 5 γ per kilo the outer leaves, 77 7 γ The drinking water contained 0 45 γ of iodine per litre

In the first experiment the thyroid-size in rats confined in the metabolism cages exceeded that in rats confined in the triple cages by 6 46   The standard error of this difference is 1 27 , ' t ' is equal to 5 1 , and the difference is, therefore, statistically significant

In the repeat experiment the thyroid-size in rats confined in the metabolism cages exceeded that in rats confined in the triple cages by 6 34   The standard error of this difference is 1 10 , ' t ' is equal to 5 8 , and the difference is, therefore, statistically significant

In the repeat experiment data were available showing the distinction by dietetic conditions , thus —

Stock diet —

Metabolism cages (11 rats)   Mean ' r ' = 18 04 ± 1 30
Triple cages (10 rats)   Mean ' r ' = 12 92 ± 0 32
Excess in the first over the second   = 5 12 ± 1 34

t = 3 8 , the difference is, therefore statistically significant

Cabbage-wheat-cholam diet —

Metabolism cages (12 rats)   Mean ' r ' = 20 87 ± 1 44
Triple cages (10 rats)   Mean ' r ' = 13 41 ± 0 75
Excess in the first over the second   = 7 46 ± 1 62

t = 4 6 , the difference is, therefore, statistically significant

The rats fed on either of the above diets and confined in the metabolism cages showed a significant excess in the size of the thyroid gland over those fed on the same diet when confined in the triple cages

These results are also expressed as frequency distributions in tabular and in diagrammatic form (Figs 1 and 2)

	FREQUENCY DISTRIBUTION OF ' R '			
	NUMBER OF RATS HOUSED IN			
Range of ' r '	Triple cages		Metabolism cages	
	First experiment	Repeat experiment	First experiment	Repeat experiment
4 0– 6 9	2			
7 0– 9 9	13	1		
10 0–12 9	20	8	6	1
13 0–15 9	13	10	8	5
16 0–18 9		1	21	5
19 0–21 9			4	4
22 0–24 9			6	5
25 0–27 9			2	2
28 0–30 9				1
TOTALS	48	20	47	23

FIRST EXPERIMENT
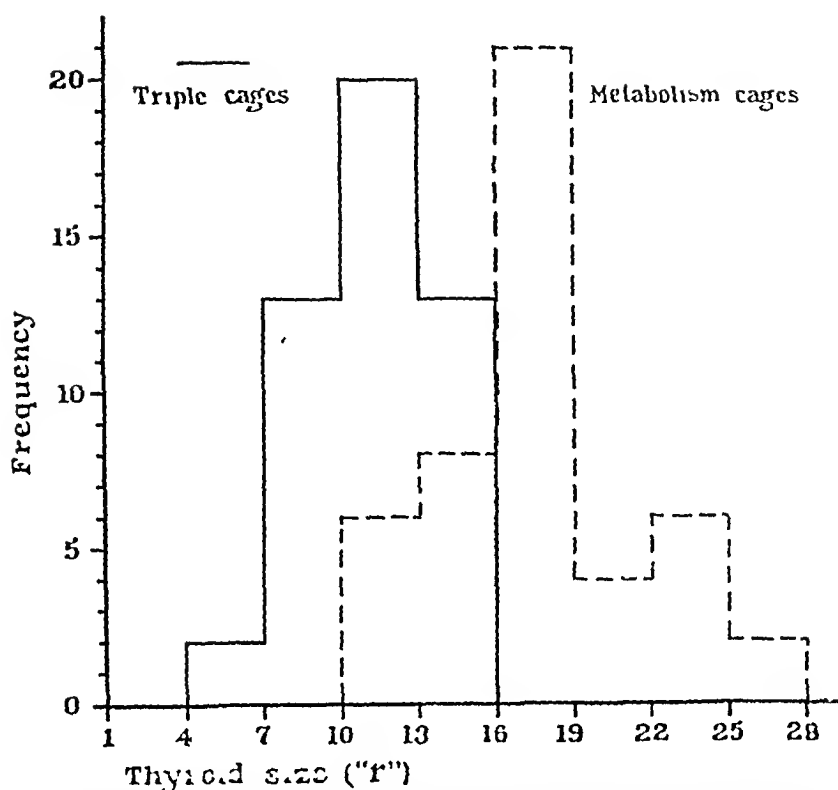
Fig 1   Frequencv distribution of thyroid sizes (i e, thyroid weights in mg per 100 g of body weight) in the first experiment   Note positive skewness of the frequency distribution diagram (indicative of the incidence of goitre) of thyroid sizes in rats confined in metabolism cages

REPEAT EXPERIMENT
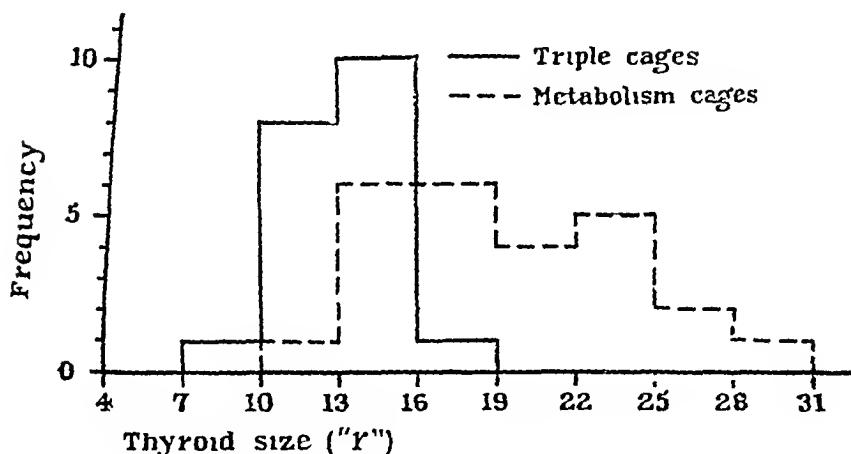
Fig 2   Frequency distribution of thyroid sizes (i e, thyroid weights in mg per 100 g of body weight) in the repeat experiment   Note positive skewness of the frequency distribution diagram (indicative of the incidence of goitre) of thyroid sizes in rats confined in metabolism cages

extracts so obtained were freed of the solvent and the residue taken up in olive oil This was administered intraperitoneally to young adult male rats in doses equivalent to 100 grammes of the lathyrus No symptoms referable to the nervous system resulted from these inoculations
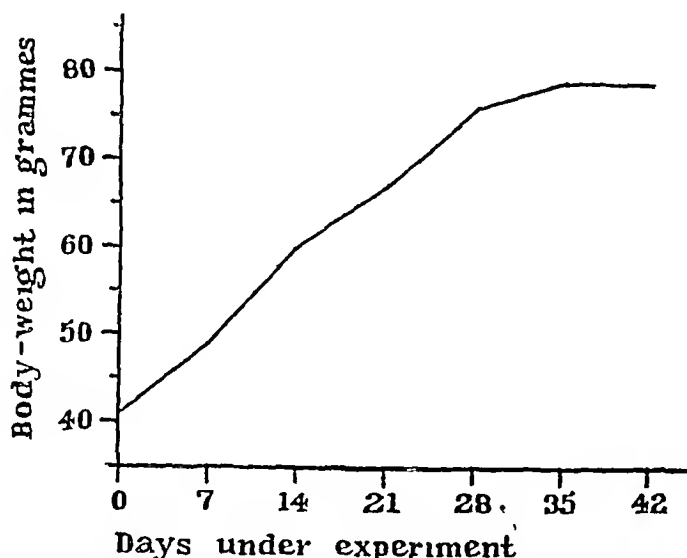
TEXT FIGURE — Composite weight curve of eleven young rats fed for 42 days on an exclusive diet of *Lathyrus sativus* and water

The result of this experiment was the same as that previously reported from these Laboratories (McCarrison, 1927) An exclusive diet of *Lathyrus sativus* failed, within a period of six weeks, to cause lathyrism or any disease of the nervous system, recognizable clinically as such, in albino rats

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EFFECT OF PLASMA FROM POLYNEURITIC FOWLS ON THE GROWTH *IN VITRO* OF EMBRYO-CHICK INTESTINE

BY

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AND

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FIFTEEN years ago attention was drawn by the senior author (McCarrison, 1919 to 1922) to the important relation that exists between the vitamin content of food and the functions of the gastro-intestinal tract   It was shown by experiments carried out on pigeons and on monkeys that faulty food deficient in vitamins gave rise to cellular changes indicative of impaired functional efficiency of such structures as the peptic glands, the glands of Brunner and those of Leiberkuhn, the mucous glands, the muscularis mucosæ, the neuro-muscular mechanism and the lymphoid tissue of the tract   Monkeys fed on antoclaved diets were prone to develop gastric and intestinal disorders and some had gastric ulcer   Guinea-pigs fed on scorbutic diets not infrequently developed duodenal ulcer   Albino rats (McCarrison, 1931), fed on certain national diets of India, were found to have ulcers of the stomach and/or duodenitis in a relatively high proportion of cases—these diets being deficient in vitamins of the A, B and C classes   It was apparent from these observations that while all these classes of vitamins had a relation to the health of the gastro-intestinal tract priority of place in this regard was to be accorded to vitamin B

The original observations referred to above were confirmed and extended by other investigators, notably by Cramer who, as recently as February of the present year, has again emphasized the importance of the relation of vitamin B to gastro-intestinal functions (Cramer, 1934)   Attention may also be directed to three other recent publications which bear on the relation of vitamin B to the functional efficiency of the gastro-intestinal tract   The first deals with the interesting and suggestive observations of Florey and Harding (1933) in regard to the functions of Brunner's glands and the pyloric end of the stomach   They have found that the secretion of

these glands is highly alkaline (pH 8), the alkalinity being due to its content of bicarbonates   The significance of this alkaline secretion for the protection of the gastric and duodenal mucosa is indicated by them, and a possible relationship between a failure of secretion and the production of peptic ulcer is suggested   The second paper relates to the production of gastric ulcers in albino rats as a result of the specific influence of deficiency of vitamin B, uncomplicated by inanition (Smie and Thatcher, 1933), and the third (Webster and Armour, 1934) provides experimental proof that ' the presence of vitamin-B complex in the body is absolutely essential for the activity of the gastric glands '   As to the fraction of the complex responsible for this activity conclusive evidence is not yet forthcoming   In regard to it these authors say   ' At present we may only state that autoclaved yeast, which presumably contains more vitamin B_2 than B_1, was effective in restoring the normal reaction of the gastric mucosa to nervous and chemical stimuli, although it took a much longer time than non-autoclaved yeast '   It is a matter of little practical importance—though of much scientific interest—whether the proper functioning of the gastric glands, and other parts of the gastro-intestinal tract, depends on an adequate supply of vitamin B_1 B_2, B_4 or B_x What is of importance is that a sufficient supply of the vitamin-B complex is essential to the normal functional efficiency of the tract as a whole   The present paper provides further evidence of this truth

Feeding Experiments

Six one-year old white leghorn cocks of the same strain were fed as follows   Three on a mixed diet of whole grains and green vegetables and three on an exclusive diet of washed, polished rice   The washing of the rice was very thorough so as to remove as much of its vitamin-B content as possible (McCarrison and Norris, 1924)   After washing, it was spread out on flat trays to dry in the sun   The birds were not artificially fed but were allowed to eat as they chose   All three developed *polyneuritis gallinarum*   one on the twenty-sixth, one on the thirty-first and the third on the thirty-second day of the experiment, the control birds, fed on the well-constituted diet, meanwhile remaining in perfect health   On the first appearance of polyneuritis the affected bird was bled from the carotid artery under strict aseptic conditions   At the same time a control bird was similarly bled   Unfortunately, one of the polyneuritic birds died under this operation   The plasmas thus collected were used for tissue-culture experiments   The pH of the plasma from control birds was 7 85, that of plasma from the polyneuritic birds, 7 80   It was observed that the plasma obtained from the polyneuritic birds was of a greenish tinge (*see* also McCarrison and Sankaran, 1933)

Tissue-culture Experiments

The hanging drop method of tissue culture was used   The media employed consisted of equal parts of the above plasmas and Tyrode solution   For the sake of brevity the two media, in which the growth of embryonic intestine is to be contrasted, are referred to as ' polyneuritic plasma ' and ' normal plasma ', no further reference to their dilution with Tyrode solution being made   The tissue used was the upper part of the intestine of the 8- to 9-day old chick-embryo   Six cultures were put up in the fresh plasma of each polyneuritic bird and six in the fresh plasma of its

Fig 2   48 hour growth in 'polyneuritic plasma' of the same embryo chick intestine as in Fig 1   Living, unstained specimen   Magnification as in Fig 1, with which compare

Fig 1   48 hour growth of embryo chick intestine in 'normal plasma'   Living, unstained specimen   Magnification as in Figs

(b) In ' normal plasma ' the growth was mainly epithelial, vast sheets of it spreading out into the medium in periods as short as 48 hours incubation (Plate V, fig 1, and Plate VI, fig 3) Such scanty growth as did occur in ' polyneuritic plasma ' was mainly fibroblastic (Plate V, fig 2) although in some specimens (Plate VI, fig 4) a restrained epithelial growth was also apparent

Forty-eight-hour cultures in ' polyneuritic plasma ', such as are illustrated in Plates V and VI, figs 2 and 4, were sub-cultured into ' polyneuritic plasma ' and into ' normal plasma ' The results of this procedure are shown in Plates VII and VIII, figs 5 to 8 They may be summarized thus :—

(1) Any growth of the original explant that had occurred after 48 hours culture in ' polyneuritic plasma ' rapidly disintegrated on the transfer of the explant to a fresh supply of this plasma, leaving at the most a few stray fibroblastic protrusions from its surface (Plate VII, fig 6, and Plate VIII, fig 8, compare with Plate V, fig 2, and Plate VI, fig 4)

(2) Forty-eight-hour cultures in ' polyneuritic plasma ' exhibited on transference to ' normal plasma ' considerable epithelial growth after 24 hours in the latter (Plate VII, fig 5 and Plate VIII, fig 7) But the sheety growths of epithelium were thin often faint, and soon became vacuolated Contact with the ' polyneuritic plasma ' for 48 hours previous to their transfer to ' normal plasma ' had apparently impaired the vitality of the cells Growth in these circumstances was mainly epithelial, though occasional cilia-like fibroblastic outgrowths were also to be seen

Conclusion.

Growth *in vitro* of intestinal epithelium of the embryo chick is greatly impaired by deficiency of vitamin B in the plasma wherein the intestinal tissue is cultured It is possible that this impairment, as illustrated in the present experiments, is not due solely to deficiency of this vitamin but depends to some extent also on other changes in the plasma brought about by subsistence on the rice diet whose major fault was one of deficiency of vitamin B

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ATTEMPTS TO PRODUCE URIC ACID CALCULI
IN ALBINO RATS

BY

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CHEMICAL analysis of human, cattle and rat stones (Newcomb and Ranganathan, 1929 , Ranganathan, 1930a, 1930b) has shown that while most human stones contain uric acid or urates (the uric acid content varying from less than 1 to over 95 per cent of the weight of the stone), cattle and rat stones are free from it   This is attributed to a difference in the physiological disintegration of purine bodies, whereby the process in man stops short at uric acid, while in cattle and rats the uric acid is further oxidized to allantoin   The latter, being several times more soluble than uric acid[*], goes into solution in the urine and does not form a constituent either of cattle or of rat stones

This conversion of uric acid to allantoin is accomplished by the enzyme, uricase, found in all animals except man and higher apes   In man the 'uricolytic index', representing the ratio of allantoin nitrogen to the sum of uric acid nitrogen and allantoin nitrogen, is low, being of the order of 34 (Hunter and Givens, 1914) , in rats it is high, being of the order of 95 or 96

The aim of the present investigation was to ascertain whether uric acid, if ingested in amounts largely in excess of that normally oxidized by uricase in rats, would appear in their urine as such , and, if so, whether the production of uric acid calculi in rats was possible   To these ends, the following experiments were undertaken —

Experiment I

Three groups of four young albino rats, of the same group body-weight, were used   Each group was made up of two males and two females   They were kept

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[*] Solubility of uric acid in 100 g of water at 20°C =0 006 g   (SEIDELL   'Solubilities of Inorganic and Organic Substances', 1917)   Solubility of allantoin 0 6 g in 100 g of water in cold (' The Chemists' Year Book ', 1925)

in individual metabolism cages, then urine being collected in flasks containing crystals of thymol   They were fed as follows —

> GROUP I   on 'stock' diet, composed of whole-wheat flour cakes (*chapatties*) smeared with butter, sprouted gram, fresh cabbage, carrots and about 10 c c of milk per rat per day   This group served as controls
>
> GROUP II   on the same diet as Group I together with five to ten grammes of fresh spleen (sheep) per rat per day
>
> GROUP III   on the same diet as Group I together with uric acid admixed with the food

The animals in Group II were given their daily dose of spleen in the morning when they were hungry, after the spleen had been eaten, they were given their food ration   Similarly, the animals in Group III were first given their daily dose of uric acid, in the form of pills made up with *chapatti*, and then their daily ration The initial dose of uric acid was 10 mg per rat per day, this was gradually increased until the animals were receiving as much as 200 mg daily

The urine voided by the four animals in each group was mixed together and the uric acid and allantoin contents of the mixture determined, the estimations always being made on fresh specimens   The uricolytic index was calculated therefrom Uric acid was estimated colorimetrically by Benedict's method   Allantoin was estimated by Christmann's method (Christmann, 1926), this was slightly modified so as to reduce the number of manipulations   Christmann's method was followed in all its details up to the point at which the mercury-allantoin precipitate was obtained, but instead of liberating the allantoin from this precipitate and converting it to oxalic acid for titration against permanganate, the nitrogen content of the mercury-allantoin precipitate was determined and the allantoin values computed therefrom.   This method gave, even with small amounts of allantoin, yields varying from about 90 to 95 per cent of the theoretical   Its accuracy was further tested on samples of urine and on urine to which known amounts of allantoin were added, good recoveries were obtained

The results of this experiment were as follows —

(1) The uric acid content of the urine of animals fed on the 'stock' diet *plus* uric acid (Group III) was not greater than in those fed on the 'stock' diet *plus* spleen (Group II)   (2) The uric acid content of the urine of the animals in Group III was only slightly greater than that present in the urine of those fed on the 'stock' diet without added uric acid (Group I), the slight increase in the former was not commensurate with the large amounts of uric acid ingested   (3) The allantoin content of the urine of rats in Group III was slightly increased, but the increase was not proportional to the amounts of uric acid ingested   (4) The uricolytic indices did not show any appreciable differences in the three groups   the values for Group III varied between 93 7 to 98 1, for Group I, between 92 9 and 97 9, for Group II, between 93 4 and 98 3

It was surmised from these results that the major part of the ingested uric acid escaped assimilation, or, if assimilated, that it was not oxidized to allantoin but to some other nitrogenous product   Accordingly, the total nitrogen content of the fresh urine was estimated   The results showed that the nitrogen content of the urine of rats in Group III was only slightly higher than that of those in Group I, while that of the rats in Group II was distinctly higher   The absence of an increase

either in uric acid or in allantoin or in total nitrogen in the urine of the animals ingesting uric acid shows that, when administered orally, uric acid is not assimilated to any appreciable extent   Determinations of the uric acid content of the fæces showed that the fæces of the animals ingesting uric acid (Group III) invariably contained more uric acid than those of the animals in the other two groups, even after allowance was made for the variations in the amounts of fæces excreted in the different groups

Experiment II

To facilitate the better assimilation of uric acid when ingested as such, the animals in Group III were given sufficient sodium carbonate along with the uric acid to form the soluble mono-sodium urate (10 mg of Na_2CO_3 to every 31 7 mg of uric acid)   The mixture of sodium carbonate and uric acid was administered in gradually increasing amounts, beginning with a dose containing 10 mg of the acid and ending with one containing 100 mg per rat per day   The animals in Groups I and II were fed as in Experiment I

The uric acid, allantoin and total nitrogen contents of the urine of the animals in the three groups were estimated and the uricolytic indices calculated therefrom The results showed that the uric acid content of the urine of rats in Group III was not increased to any considerable extent in consequence of the ingestion of a mixture of uric acid and sodium carbonate , the allantoin content did show an increase, but the increase was not proportional to the amounts of uric acid ingested   Sodium carbonate did not, therefore, favour the assimilation of uric acid , an observation confirmed by the relatively poor nitrogen content of the urine of animals in Group III

Experiment III

While continuing to feed the animals in Groups I, II and III in the same way as in the previous experiments, those of Group III were given subcutaneous and intravenous injections of an isotonic solution of uric acid   The initial dose of uric acid injected into the animals of Group III was 2 mg , this was gradually increased up to 96 mg per rat per day   Estimations of the uric acid, allantoin and total nitrogen contents of the urine of animals in the three groups were made   The results showed that the intravenous or subcutaneous injection of uric acid did increase the uric acid, allantoin and total nitrogen contents of the urine , but this increase was not in proportion to the amounts of uric acid injected   Thus, the maximum amount injected (96 mg into each of four rats) caused an increase in the uric acid content of the urine which averaged barely 2 mg more than that in the controls not receiving injections of this acid   The volume of urine voided by the animals receiving injections of uric acid was not appreciably different from that of the controls not receiving injections of this acid

Experiment IV

The next step in the investigation was to administer phenyl cinchonic acid (atophan) orally with subcutaneous injections of uric acid to the animals in Group III, while those in Groups I and II were fed as in the previous experiments   (Atophan

is said to mobilize the blood and tissue uric acid in man and to get rid of it in the urine, it was thought that it might have a similar action in rats) As a result of the administration of atophan it was found that there was on the average, nearly three times as much uric acid excreted per day in the urine of the animals receiving it as in that of the controls. This increase in uric acid was accompanied by a corresponding increase in the volume of urine voided, so that the percentage concentration of uric acid in the urine was not materially altered. There was no difference in the values of the uricolytic indices in the three groups

Uric acid content of blood of rats

Conflicting statements appear in the literature regarding the uric acid content of the blood of albino rats. Folin and Morris (1913) found as much as 2 mg to 3 mg per 100 c c of blood. Anderson *et al* (1930) give 1 86 mg per 100 c c of blood as the mean value with variations ranging from 1 35 mg to 2 40 mg. Schamberg and Brown (1923) have found only traces of it in rat's blood. As the experiments described in the preceding pages afforded material for an examination of the blood uric acid the animals were anæsthetized at the close of the experiment, and as much blood as possible removed from the heart. Animals that survived more than one such bleeding were used for duplicate and triplicate estimations. Uric acid was determined colorimetrically in the protein-free filtrate of the blood. It was found as a result of several estimations that the uric acid content of rat's blood varied from 1 54 mg to 3 09 mg with a mean of 2 26 mg per 100 c c and that there was no increase consequent on prolonged feeding either on a glandular organ (spleen) or on uric acid

Summary

(1) Although realizing that rats have a high uricolytic index, attempts were made to increase the uric acid concentration of their urine, both by the oral administration of uric acid and by feeding them on a glandular organ (spleen)

(2) The uric acid ingested orally did not lead to an increase in the excretion either of uric acid or of allantoin or of total nitrogen, nor was there an appreciable increase when the uric acid was administered with sodium carbonate in amounts sufficient to form the mono-sodium urate

(3) Intravenous and subcutaneous injections of an isotonic solution of uric acid did increase the uric acid, allantoin and total nitrogen contents of the urine, but the increase was not commensurate with the amounts of uric acid injected

(4) Intravenous and subcutaneous injections of uric acid followed by the ingestion of atophan led to a greater increase in the daily excretion of uric acid in the urine, the volume of urine voided was also increased so that the percentage concentration of uric acid in the urine was not materially altered

(5) Uric acid was found to be present in the blood of rats in amounts ranging from 1 5 mg to 3 1 mg per 100 c c, neither the oral nor the subcutaneous nor the intravenous administration of uric acid increased the level of this acid in the blood

Conclusion

The above observations appear to indicate that the experimental production of uric acid calculi in albino rats is not possible

This investigation was carried out under the direction of Sir Robert McCarrison to whom I am much indebted for help and criticism

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PLAGUE INQUIRY IN THE CUMBUM VALLEY, SOUTH INDIA

BY

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INTRODUCTION

A RAT-FLEA survey of the Madras Presidency was commenced in 1928 and the results have been published in a series of thirteen regional reports and a summary (King and Pandit, 1931) A geographical survey of plague in the Presidency was compiled by the former Director of Public Health (Russell, 1930) As a sequel to these studies it was suggested by Dr C G Pandit that the problem of plague recrudescence might profitably be investigated in the Cumbum Valley Accordingly the senior writer, who had been connected with the flea survey work, was appointed for this special duty and commenced field observations in May 1930 His expenses have been met largely by the Indian Research Fund Association and he has worked under the administrative control of the Director of Public Health Lieut -Colonel H H King, I M S , Director, King Institute, who has been in close touch with this work from the commencement, and we have to thank him for much valuable advice as well as for the loan of personnel and laboratory appliances Lieut -Colonel J R D Webb, I M S , Director of Public Health, has spared no effort in securing facilities for the field workers and has successfully obtained the co-operation of various local bodies His visits to the field laboratory, often in company with other medical men or administrative officers, were a source of great encouragement

METHODS

The technical methods adopted are largely those of the Haffkine Institute as detailed in a paper compiled by one of the authors (Webster, 1932) and need not be repeated here The main facts regarding the identification of the rodents have also been collected and published elsewhere (Webster, 1933) The fleas encountered have belonged to a very few well-known and easily-identified species A recent improvement in methods is the recognition that the bone marrow is one of the most satisfactory sources of plague bacilli in plague-infected carcasses especially when putrefaction has commenced (Reynal and Wassilieff, 1933)

TOPOGRAPHY

The Cumbum Valley is part of Periyakulam taluk of the Madura district Cumbum village, where the field laboratory was established, is about Lat 9° 44′ N and Long 77° 17′ E   The valley is 35 miles long from Theni to Kumili   it h

MAP

CUMBUM VALLEY

Scale 12 miles to 1 inch
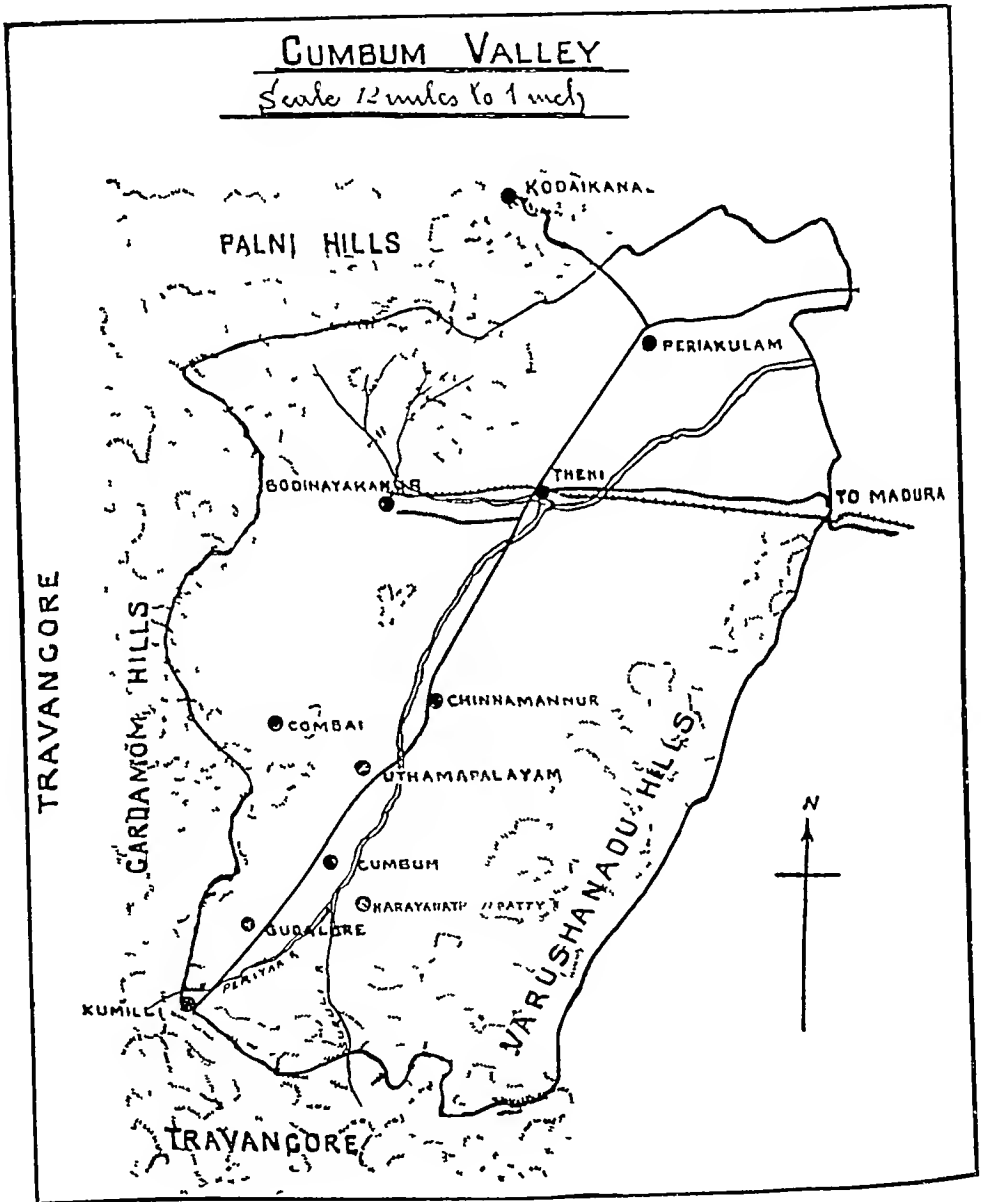
20 miles wide near Theni, while it narrows in the South-West to six miles at Gudalur   On the West are the Cardamom Hills of the Western Ghats and on the East and South-East the Varushanad Hills   These have an altitude of about

4,000 feet above sea-level with peaks reaching to 6,000 feet or more   The steep slopes of these hills form a natural barrier which is cut by only one road, that which passes into Travancore at Kumili   Them is about 1,000 feet above sea-level and the valley slopes up to about 1,450 feet at Gudalur   In the centre of the valley the land is wet and fertile being irrigated by perennial streams   The high sides of the valley consist of dry Madras red soil, partly cultivated under wells   Limestone is generally met at a depth of two or three feet and rock appears in places

<div align="center">CLIMATE</div>

No official records are kept of climatic conditions in the Cumbum Valley except for the rainfall at Uthamapalayam   In general the climate is equable and extreme heat is avoided by reason of the cool breezes from the high lands to the West   We have now a three years' record of the daily maximum and minimum wet and dry bulb thermometer readings taken in the field laboratory in Cumbum village   These were not taken under standard conditions, i e , in the Stevenson screen, but in a mud-walled, straw-roofed building   Table I shows average monthly figures compiled from these readings, along with the rainfall figures for Uthamapalayam   The saturation deficiency has been calculated from 8 a m readings only   The mean monthly temperature has varied from 85 7°F in April 1933 to 72 8°F in January 1932   The highest mean daily range was 20 8°F in March 1931 and the lowest 6 0°F in September 1933   As regards saturation deficiency it appears that the spring of 1931 was distinctly more dry than that of the two following years   The average rainfall, according to the official records, is 27 42 inches, more than half of which occurs during the North-East monsoon in the last three months of the year   Rain occurs almost every month and the countryside rarely assumes the familiar burnt-up hot weather appearance —

<div align="center">TABLE I</div>

<div align="center">*Meteorological data, Cumbum, 1931–1933*</div>

Year and month	Mean tempe rature °F	Mean range °F	Mean S D, inches	Rainfall, inches	Rainy days
1931					
January	74 3	14 5	0 17	0 07	2
February	77 3	18 5	0 23	Nil	Nil
March	84 4	20 8	0 35	1 15	1
April	84 5	15 7	0 34	8 97	4
May	85 4	14 0	0 33	1 99	2
June	81 3	9 5	0 35	0 19	1
July	79 7	9 7	0 28	0 44	2
August	77 5	7 7	0 23	3 24	16
September	78 7	10 9	0 26	0 93	5
October	79 0	11 8	0 27	4 69	10

TABLE I—*concld*

Year and month	Mean temperature, °F	Mean range °F	Mean S D, inches	Rainfall, inches	Rainy days
1931					
November	76 7	10 5	0 10	9 03	13
December	74 6	8 6	0 11	9 22	12
1932					
January	72 8	14 4	0 10	*Nil*	*Nil*
February	76 8	15 2	0 22	0 66	2
March	76 3	9 2	0 28	0 06	1
April	84 4	15 8	0 31	2 35	5
May	81 2	10 9	0 18	5 49	12
June	81 2	9 6	0 31	2 35	5
July	79 1	7 7	0 25	0 86	11
August	80 0	10 9	0 33	4 96	15
September	80 1	8 5	0 23	0 56	6
October	79 8	6 3	0 18	6 09	20
November	79 1	11 5	0 14	6 81	13
December	74 4	10 2	0 11	0 77	6
1933					
January	75 7	11 8	0 18	Vil	*Nil*
February	78 8	16 3	0 24	*Nil*	*Nil*
March	82 8	17 5	0 21	1 20	3
April	85 7	14 8	0 23	1 31	5
May	82 5	12 3	0 15	2 93	13
June	79 7	9 9	0 20	1 74	9
July	78 8	9 1	0 25	3 62	10
August	79 3	10 2	0 30	5 33	15
September	79 4	6 0	0 31	3 61	11
October	77 7	12 9	0 14	10 80	17
November	77 0	13 5	0 13	7 52	9
December	75 4	14 1	0 13	0 45	3

LOCAL CONDITIONS

The population of the valley is about 300,000, living in 50,000 houses There are about 60 villages mostly with 2,000 to 10,000 inhabitants Per square mile there are 396 persons (Periyakulam taluk, 1931 census) Most of the people are Hindus, chiefly of the lower castes, Tamil is the mother tongue of more than half the population and this is the common language of the valley Telugu and Kanarese are also quite common Most of the houses are built of mud and are generally roofed with straw thatch, but sometimes with galvanized iron Grain is stored in the houses in earthenware pots or in basket-work containers plastered with cow-dung The well-to-do farmers and merchants may have a mud-walled granary attached to their houses Some grains, like cholam, are buried in large pits outside

The only industry is agriculture The people are almost entirely employed in the cultivation of land or the tending of flocks, and wages are low One peculiarity is that the large flocks of cattle and goats are driven into the villages at dusk for safety from the Kallans, a caste whose hobby is cattle theft For this reason the fields are deserted at night and there is gross overcrowding in the villages As often as not the cattle share the human habitations Matters of hygiene are completely ignored

Rice, cholam, ragi and samai are the chief produces but cotton, gram and tobacco are also grown in places and there is a large variety of minor crops Grain is exported to other parts of the Madura District and to Travancore via Kumili Several markets (shandies) are held weekly and large quantities of grain are transported from place to place in consequence Theni weekly market is one of the largest in the Presidency and may attract as many as 1,000 bullock carts Until a few years ago there was practically no traffic in this area except by bullock cart The railway from Madura through Theni to Bodinayakkanur was opened in 1929 and there is also a regular motor-bus service on the roads from Theni to Gudalur, Bodinayakkanur, Periyakulam and Madura

HUMAN PLAGUE IN THE CUMBUM VALLEY

Indigenous cases of human plague were not detected in the Madura District until 1910, although for ten years before that many parts of the Presidency including the neighbouring district of Coimbatore had suffered severely from plague It was ten years later before human plague infection was recognized in the Cumbum Valley The history of the first cases in 1920 cannot now be traced Table II shows the reported cases and deaths for the last eight years These are the quarterly totals of the figures from the various villages of the valley

It will be understood that the diagnosis of plague is rarely made by a medical man and that there must be many errors of omission and commission Thus, the case mortality for the year is 39 per cent but that for the last quarter of the year is lower than that for the other quarters This is believed to be due to the inclusion of some cases of malarial fever among the ' cases ' On the other hand mild attacks must often go unrecorded Human plague figures are available from half a dozen sources but these do not agree Sometimes imported cases are shown separately The date under which a case is reported may not be the same in the different records This explains certain discrepancies in this paper

It is clear that there is much less plague in the second quarter of the year and the figures for that period are shown in greater detail in Table III   It cannot be said that there is a definite off-season for plague, for, as often as not, there are numerous cases in every month of the year   Only once in the eight years has a period of two months passed with no recorded death from plague   The seasonal incidence will be discussed more fully later

<div align="center">

TABLE II

Human plague in Cumbum Valley

</div>

Quarter year	1		2		3		4		Whole year	
	Cases	Deaths	Cases	Deaths	Cases	Deaths	Cases	Deaths	Cases	Deaths
1926	160	83	17	11	226	100	97	40	500	234
27	135	34	140	62	783	297	1,281	490	2 339	883
1928	530	246	39	22	260	95	695	274	1,524	637
1929	375	176	125	41	142	53	270	113	912	383
1930	394	160	115	49	296	139	299	167	1,104	515
1931	556	287	7	3	382	178	215	61	1,160	529
1932	123	45	183	68	699	278	1,009	310	2,014	701
1933	1,004	265	24	10	337	101	273	107	1,638	483
TOTALS	3,277	1,296	650	266	3,125	1,241	4,139	1,562	11,191	4,365
Percentage of mortality	39		40 9		39 7		37 7		39	
Percentage of cases	29 3		5 8		27 9		37 0			
Percentage of deaths	29 7		6 1		28 4		35 8			

TABLE III

Plague in the second quarter

Year	APRIL		MAY		JUNE	
	Cases	Deaths	Cases	Deaths	Cases	Deaths
1926	8	8			9	3
1927	4	2	39	18	97	42
1928	9	6			30	16
1929	5	4	72	26	48	11
1930	29	11	44	13	42	25
1931					7	3
1932	19	9	26	13	138	46
1933	4	3	3		17	7

RAT-FLEA SURVEY

The rat-flea survey of the Cumbum Valley by one of us (P V G) in 1928-1929 (King *et al*, 1929) showed the following data —

	Rats examined	*cheopis* index	*asta* index
Uthamapalayam	233	4 69	2 30
Theni	202	2 00	4 87

It was noted that in Theni the proportion of *cheopis* to all rat fleas was much higher in cotton godowns than elsewhere

The results of a continuous rat-flea survey over a period of three years are now collected in Table IV   The figures refer entirely to *Rattus rattus*   White-bellied, black and brown varieties were all numerous but the majority were brown   No attempt has been made to distinguish them either as regards flea infestation or plague infection   The numbers of rats caught per hundred traps set have been omitted   There are many variable factors affecting such figures apart from the size of the rat population   It is certain, however, that house rat infestation is a marked feature of life in the villages   One point of interest is that of 7,143 rats trapped singly, no less than 4,485 or 62 8 per cent were females   Similar figures were obtained in the great majority of the regional rat-flea surveys of the Presidency This is contrary to the experience of certain French authorities who have recently condemned trapping as an anti-rat measure because more males than females are caught and breeding actually increases (Loir, 1933)

As regards the fleas, only *cheopis* and *astia* need be considered Actually 136 *Echidnophaga gallinacea* and four *Ctenocephalides* sp were also obtained The gross flea-index has varied from 7 0 in September 1933 to 2 6 in April 1933, the average being 4 3 Generally speaking there were more fleas per rat in the cold weather Reference to the figures for the specific flea-indices shows that the change in the index from month to month affected chiefly the *cheopis* population The index for *astia* has varied from 2 1 to 0 6 but has usually been between 1 0 and 2 0 The *cheopis* index, on the other hand, has varied from 5 1 to 1 4 The figure between March and August inclusive has not been above 3 0

The sex of the identified fleas was noted and according to the totals the females amounted to 41 5 per cent in the case of *cheopis* but 54 8 per cent in the case of *astia* This difference between the two species of *Xenopsylla* has been noted on several occasions by different observers but has not been explained No attempt has been made to distinguish the type of premises in which the rats were trapped In the villages of the Cumbum Valley there is no separation of residential and commercial areas

TABLE IV

Rat-flea survey, Cumbum Valley, 1931–1933

Year and month	Number of R rattus	Number of fleas	Total flea index	cheopis index	astia index	Human plague deaths
1931						
January	380	1,870	4 9	3 9	1 0	165
February	289	1,970	6 8	5 1	1 7	90
March	337	1,175	3 5	2 1	1 4	32
April	265	984	3 7	2 2	1 5	0
May	153	441	2 9	1 5	1 4	0
June	215	655	3 1	1 4	1 6	3
July	184	655	3 6	2 1	1 4	7
August	154	618	4 0	2 9	1 1	71
September	178	932	5 2	3 6	1 7	100
October	202	1,089	5 4	3 4	2 0	32
November	196	918	4 7	3 4	1 3	8
December	208	828	4 0	2 7	1 3	21

TABLE IV—*concld*

Year and month	Number of R rattus	Number of fleas	Total flea index	cheopis index	astia index	Human plague deaths
1932						
January	297	1,655	5 6	4 4	1 2	30
February	297	1,194	4 0	2 8	1 2	12
March	50	157	3 1			3
April	167	494	3 0			9
May	65	262	4 0	2 3	1 7	13
June	207	636	3 1	2 0	1 0	46
July	303	1,158	3 8	2 9	0 8	91
August	380	1,336	3 5	2 7	0 8	59
September	154	763	5 0	3 7	1 2	128
October	239	1,172	4 9	3 9	1 0	58
November	193	956	5 0	4 3	0 7	104
December	215	765	3 6	2 7	0 9	148
1933						
January	264	1,010	3 9	3 2	0 7	129
February	86	305	3 5	2 5	1 1	105
March	145	412	2 8	2 3	0 6	31
April	229	590	2 6	2 0	0 6	3
May	267	924	3 5	2 6	0 9	0
June	52	224	4 3	2 7	1 6	7
July	113	345	3 0	1 9	1 1	22
August	120	481	4 7	3 0	1 7	60
September	204	1,261	6 2	4 9	1 3	19
October	70	386	5 5	4 1	1 4	18
November	106	384	3 6	2 3	1 3	27
December	159	625	3 9	2 2	1 6	62
TOTALS	7,143	30,660	4 3			1,713

Climate, flea prevalence and plague incidence having been discussed, the relationship of these may now be considered by reference to the graphs (Graphs 1, 2 and 3)

The most striking feature is the trough in the wave of plague incidence each year, most marked in April and May   In 1931 it extended from March to July inclusive   In 1932 the wave commenced to rise again in June   In 1933 it dropped abruptly in March and the autumn rise in August was comparatively slight

The *cheopis* index was, in general, lower from March to August than during the rest of the year   The *cheopis* index and plague incidence are not so closely associated, however, as appears at first glance   Thus, in 1931 the decline of plague in February preceded the fall in the *cheopis* index while in the autumn epidemic the plague incidence increased prior to the increase in fleas   In 1932 the order of events was the same but to a less marked extent   In 1933 the high plague incidence in the spring was not associated with a corresponding rise in the flea-index figure, while the autumn rise in the *cheopis* index was not accompanied by an increased incidence of plague   The evidence is, therefore, not clear that the number of human cases of plague depends directly on the number of *cheopis* fleas, as measured by the flea-index   Considering that the death of many rats from plague must release a corresponding number of fleas it is surprising that the flea-index on the surviving rats does not rise to a more marked degree

Comparing climatic conditions and plague cases it can be seen that in 1931 the end of the epidemic corresponded very closely with the period during which the mean temperature and saturation deficiency reached the range calculated by St John Brooks to be unfavourable to epidemic plague, namely, over 80°F and over 0.3 inches respectively (Plague Research Commission, 1917)   In 1932, however, plague declined to almost nil long before the temperature and humidity had reached the critical limits   In 1933 the saturation deficiency remained low but plague incidence dropped abruptly as the mean temperature rose above 80°F

It appears that conditions in the Cumbum Valley in the spring months are unfavourable for the spread of plague   It is reasonable to suppose that, when this is so, very small, and perhaps strictly local, variations in certain factors such as rat and flea prevalence may determine the appearance of human plague   As will appear later, plague infection in rats and fleas was often detected when human plague was absent

The *cheopis* index in the Cumbum is high all the year round   Hirst and others consider that a *cheopis* index of 1.0 is sufficient for the spread of plague when other conditions are favourable   The *astia* population here is of little importance, the index being low   The important result of hot dry weather is probably the effect on plague-infected fleas   The distinction between ' infected ' and ' infective ' has not been sufficiently emphasized   Even under the most favourable conditions only in occasional infected flea becomes capable of infecting   Transmission experiments in Bombay showed clearly that fewer infected fleas became capable of infecting in the hot season   This probably means that more infected fleas become ' blocked ' when conditions are favourable   The actual *cheopis* index is of less importance, provided it is above a certain limit, probably about 1.0

GRAPH 1

TEMPERATURE, SATURATION DEFICIENCY, X CHEOPIS INDEX AND PLAGUE DEATHS 1931
CUMBUM VALLEY

GRAPH 2

TEMPERATURE, SATURATION DEFICIENCY X CHEOPIS INDEX AND PLAGUE DEATHS 1932
GUMBUM VALLEY
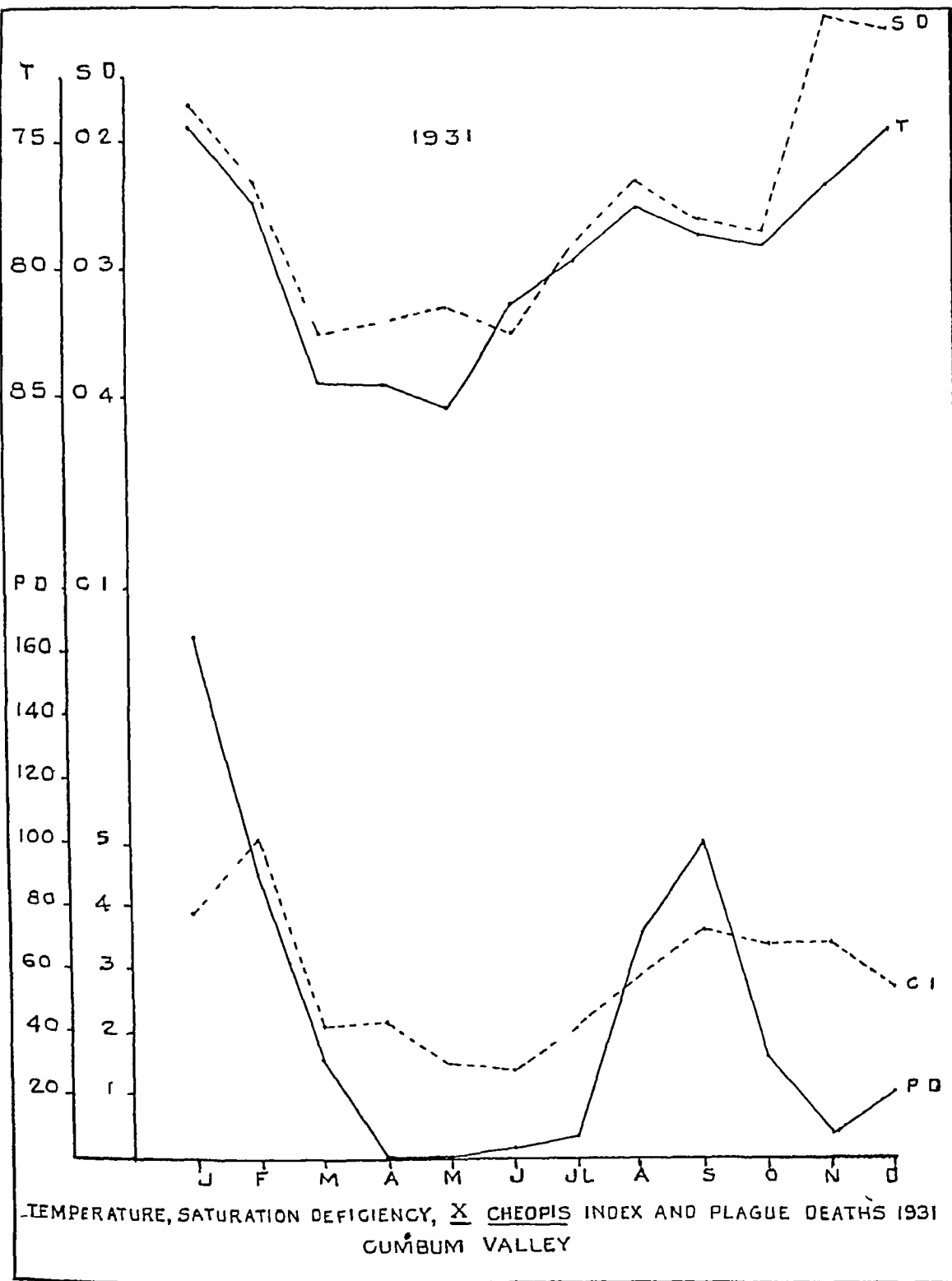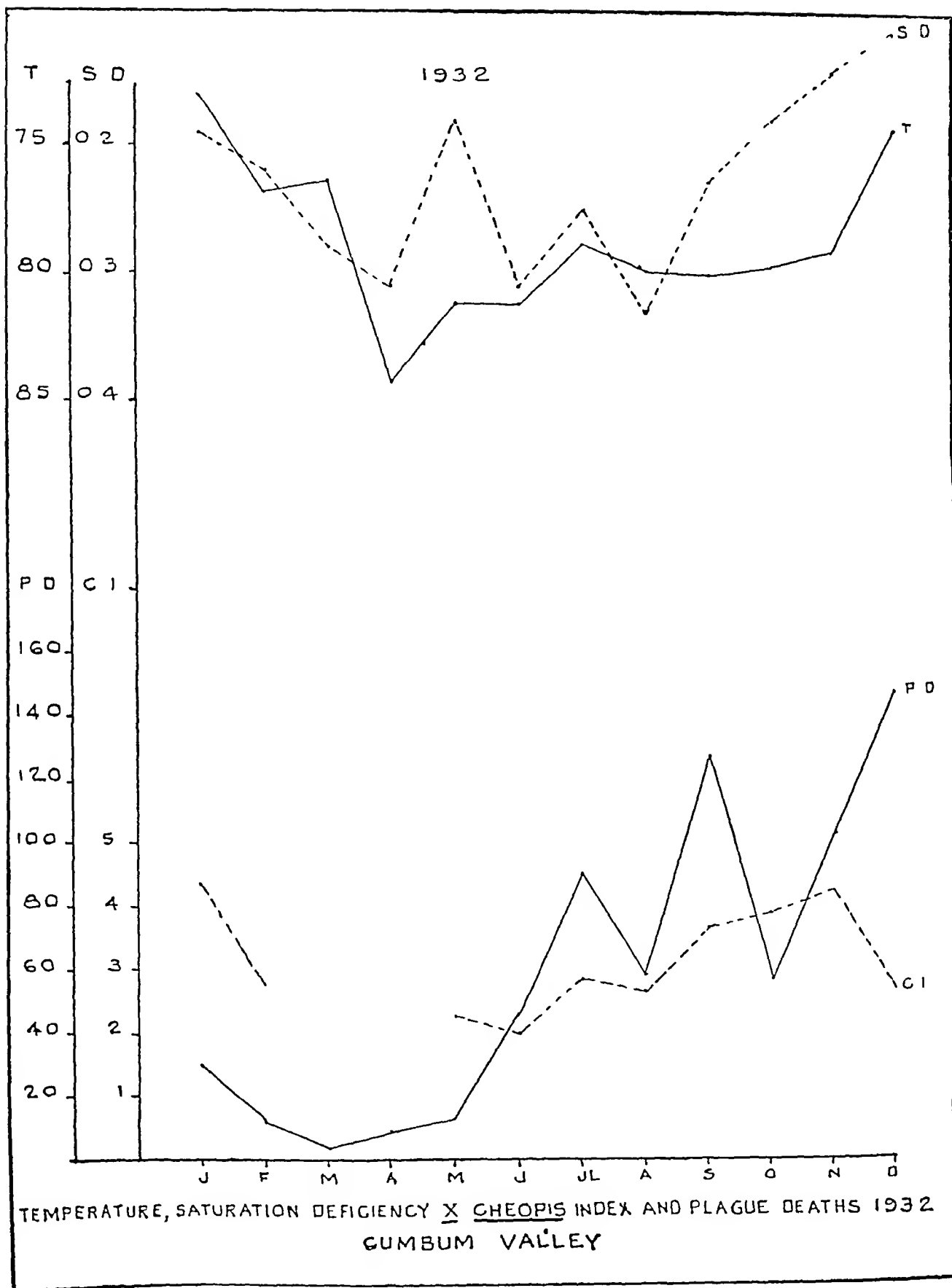
Note—The total flea index in March and April 1932 was 3 1 and 3 0 respectively The *cheopis* index was presumably about 2 0

GRAPH 3

1933

TEMPERATURE SATURATION DEFICIENCY X CHEOPIS INDEX AND PLAGUE DEATHS 1933
CUMBUM VALLEY
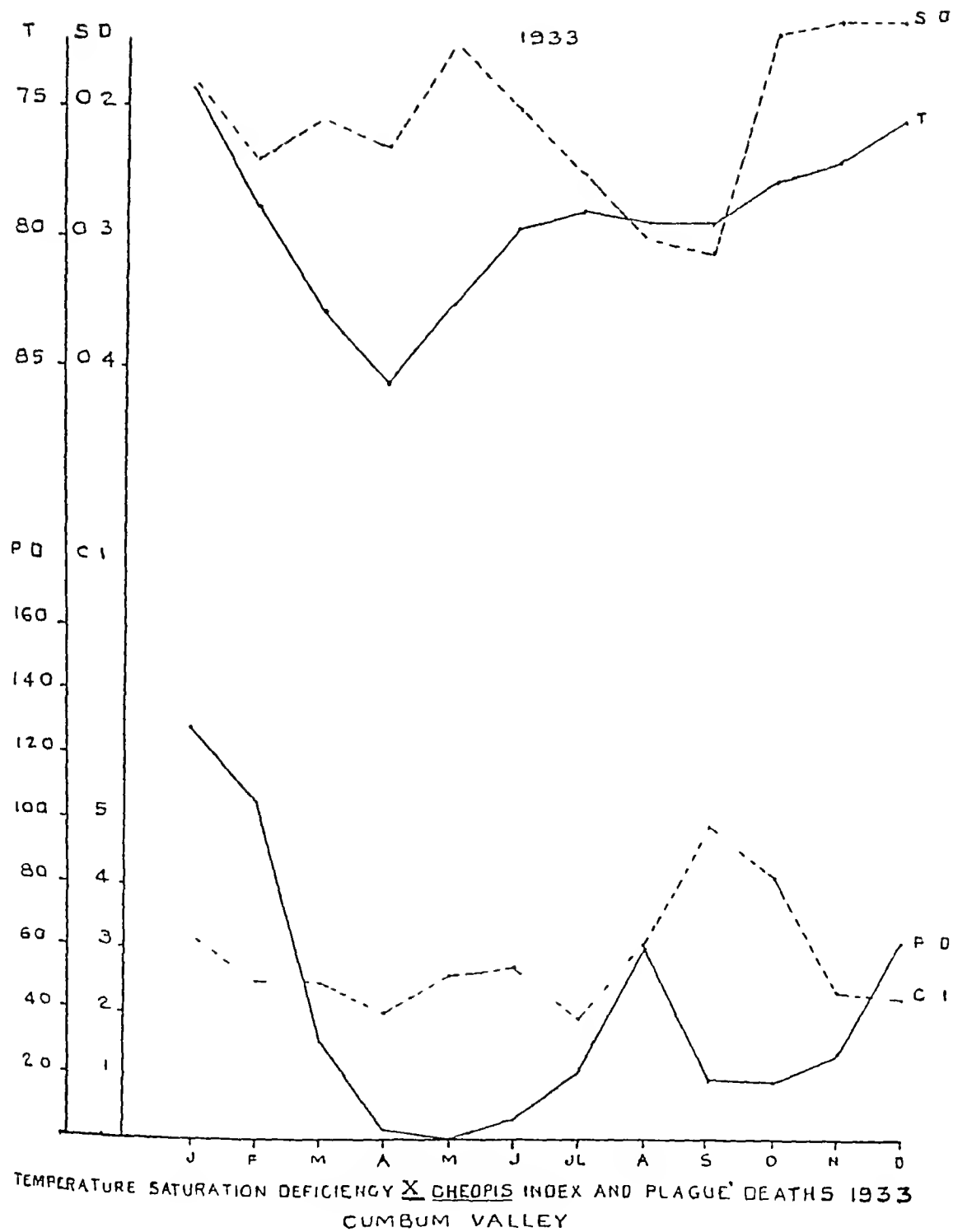
OBSERVATIONS ON THE LONGEVITY OF PLAGUE-INFECTED FLEAS

Commencing in November 1930 attempts were made to collect wandering fleas from houses and rat burrows   In the case of houses, de-flead guinea-pigs in pairs were allowed to run loose overnight   Individual burrows were tested by introducing a de-flead rat, tethered by a piece of wire about four or five feet long, and left in the burrow overnight   These tests were carried out chiefly in vacated houses   When possible the test was repeated a week later, and occasionally on several successive weeks

Altogether 196 tests were carried out in 130 houses   A total of 1,196 fleas was recovered from guinea-pigs and 2,018 from tethered rats   The fleas obtained were tested for plague infection either culturally or by injecting an emulsion of the fleas into Madras rats   Usually the whole catch from one house or burrow was emulsified and a part of the emulsion was injected into two or more rats   The animals used to trap the fleas were also isolated for observation, after being de-flead

A house or burrow was regarded as plague-infected when either the animal used as a flea trap died of plague or the tests for plague infection of fleas were positive   A note was kept of the period elapsing between the occurrence of former rat falls and the detection of plague infection

Out of the 196 tests, 41 were positive for plague infection   The fleas were a mixture of *cheopis* and *astia*   these were not tested separately   In one case the test for plague infection was positive, 13 weeks after the occurrence of a rat fall   On seven occasions there were positive results three to five weeks after the rat falls   The remaining 33 positives were within 20 days of the rat fall

In many of these cases there was no outward evidence of the presence of plague infection   A good example of this is the village of Narayanathevanpatty   There were 45 cases of plague there in March 1931, the last case being on 28th March   No further case occurred but on 28th April, one month later, two houses were found to be infected by the wandering flea test   No human plague was detected until 26th August   No rat falls had been noted in the interval

On considering these findings the question arose whether these wandering fleas were in a condition of starvation or were obtaining at least occasional feeds from surviving rats or other hosts   It, therefore, seemed desirable to obtain some more definite evidence regarding the longevity of starved plague-infected fleas

On the suggestion of Lieut.-Colonel H H King, I M S , a mud house with two rooms, rendered rat-proof with wire netting, was built in February 1932   Rats were allowed to run loose in the rooms and by the middle of March had made several deep burrows   Fleas were then released, 562 in the control room and 568 in the room to be infected   These were a mixture of *cheopis* and *astia* in more or less equal numbers   Twenty artificially-infected rats were then released in one of the rooms   After these rats died off, the infected room was tested by adding six normal rats   These promptly died of plague   The last rat died in the infected room on 10th May and ten days later the rats were removed from the control room   To detect the escape of fleas a number of guinea-pigs was kept in a mud enclosure in front of each room   None of these died of plague but an occasional flea was recovered from them   From 21st July, at weekly intervals two rats were added to each room, left for 48 hours and then recovered by trapping, and kept for observation   One of the rats removed from the control room on the first occasion died of plague

eight days later, the infection being confirmed by animal passage   It is supposed that the control room became infected by the passage of fleas through cracks in the mud wall   There is, however, an element of doubt about this result   No further evidence of plague infection was obtained

This experiment was repeated at the end of October using both rooms as infected   Over 500 fleas, a mixture of *cheopis* and *astia* caught wild, were released in each room   Six rats were then added to each room on three successive days (18 rats in all) all infected subcutaneously with saline emulsions of the spleens of highly septicæmic plague rats   When all the infected rats had died, three normal rats were added to test the infection of the rooms and these died of plague   Tests of infection of starved fleas were then carried out with the following results   Colour marking of the test rats was used to distinguish those added at different times   It was not always possible to recover every test rat but the date of death could be determined by the use of test baits, and also by means of test boards with a layer of fine sand to show up foot prints and tail tracks —

Room I	Room II
8th November, 1932	7th November, 1932
Last rat died	Last rat died
37 fleas examined	35 fleas examined
28 *cheopis*	24 *cheopis*
18th November, 1932	18th November, 1932
Ten days starvation	11 days starvation
Test rats added	Test rats added
Result positive	Result positive
24th November, 1932	26th November, 1932
Last rat died	Last rat died
8th December, 1932	8th December, 1932
14 days starvation	12 days starvation
Test rats added	Test rats added
Result positive	Result positive
15 fleas examined	43 fleas examined
13 *cheopis*	11 *cheopis*
16th December, 1932	13th December, 1932
Last rat died	Last rat died
30th December, 1932	30th December, 1932
14 days starvation	17 days starvation
Test rats added	Test rats added
Result positive	Result positive
12th January, 1932	9th January, 1932
Last rat died	Last rat died
4th February, 1933	30th January, 1933
23 days starvation	21 days starvation
Test rats added	Test rats added
Result positive	Result doubtful
Two rats survived	18 fleas examined
116 fleas examined	1 *cheopis*
101 *cheopis*	
24th June, 1933	20th June, 1933
One decomposed carcass	No rats were recovered
	One guinea pig at entrance
	died of plague

Plague infection by fleas after periods of starvation of 10, 11, 12, 14, 17, 21, and 23 days has, therefore, been demonstrated   The death of the guinea-pig in connection with the second room after a period of several months is not emphasized, as it is not known for certain when the rats died   It appears that *cheopis* fleas were in the majority in the first room

These experiments have again been carried out under somewhat different conditions   A series of five mud huts was built six feet apart   These were made rat-proof by an inner lining of wire netting and a rat-proof ledge prevented the chance of stray rats climbing up the outside   Normal rats were allowed to burrow in the huts for a month   About 250 fleas caught wild in Cumbum, a mixture of *cheopis* and *astia* were added to each hut   On 23rd May, 1933, six rats infected with the same strain of plague were added to each hut and this was repeated a week later   The original healthy rats died of plague   When the rats were believed to have died, as shown by test baits and test boards, the fleas were left to starve and susceptible normal rats were added after certain intervals   As can be seen from Table V, fleas were able to transmit infection after periods of starvation of 6, 14, 22 and 29 days —

<p align="center">TABLE V</p>

Hut —	3	5	6	4	7
Last infected rat alive	4-6-33	6-6-33	6-6-33	13-6-33	16-6-33
Test rats added	10-6-33	20-6-33	23-6-33	5-7-33	15-7-33
Period of starvation of fleas, days	6	14	17	22	29
Test rats added	3	6	3	6	6
Test rats infected with plague	2	2		2	2

Of these positive test rats, one in hut 4 and one in hut 5 showed signs of resolving plague but were negative on animal passage   Two rats from hut 7 showed similar lesions but gave positive confirmation   In hut 7 one of the original normal rats caught on 16th June, 1933, died seven days later with signs of resolving plague, again confirmed by the usual tests

When there were no longer live rats in the huts they were left undisturbed for further periods and tested as before   Huts 4, 6 and 7 were negative after 21, 20 and 33 days respectively   In hut 3, tested after ten days starvation of fleas, there were two deaths from acute plague, while one of the six test rats showed lesions of resolving plague but infection was not confirmed   This same hut after a further 16 days starvation of fleas again gave a positive result, one of the six test rats showing evidence of resolving plague, the infection being confirmed

,   Altogether there were seven cases of resolving plague, four of which gave confirmation of plague infection on animal passage   As no such lesions have been encountered in Madura rats infected cutaneously or subcutaneously with spleen

PLATE IX

Field laboratory, Cumbum village, showing transmission huts

emulsions there is a suggestion of reduction of virulence in the starved fleas   This is more probable than that the infecting dose from the fleas was less than usual

Another case of resolving plague confirmed on passage was seen in a Madura rat 57 days after it had been infected with an emulsion of two *cheopis* fleas from hut 3   This was the only positive result in 15 experiments with fleas from the huts   In all these cases of resolving plague the result of examining smears of heart blood and spleen was negative   This was the finding of the Plague Research Commission (1907)

SUSCEPTIBILITY OF *R rattus* TO PLAGUE

The high susceptibility of Madras rats to plague is well known   Generally the rats of an area are more highly immune to plague the more that area has suffered from plague   A comparison of the susceptibility of Madras, Madura and Cumbum Valley rats was carried out in 1930 with the results shown in Table VI   These rats were infected cutaneously or subcutaneously and the test dose in the latter case was 0 003 g of the spleen pulp of a rat recently dead of plague and showing a heavy septicæmia   Cumbum Valley rats appear to be quite as susceptible to plague as Madras rats   The small number of rats from Madura town were also highly susceptible —

TABLE VI

Susceptibility to plague of Madras, Cumbum and Madura rats

Mode of infection	RATS					
	MADRAS		CUMBUM		MADURA	
	Number	Died of plague	Number	Died of plague	Number	Died of plague
Cutaneous	104	97	234	228	14	14
Subcutaneous	100	86	86	84	21	20

OTHER ANIMALS AND THEIR FLEAS

In the course of the trapping operations in villages *Rattus rattus* was by far the commonest rodent captured and this species alone has been considered for flea-index purposes   Bandicoots (*Bandicota malabarica*) and field rats (*Gunomys kok*) have occasionally been trapped in habitations   Other rodents have been trapped from time to time away from houses and as far as possible these were searched for fleas   Only a proportion of the total catch was available for flea-counts because sometimes more than one species was caught in the same trap   Some interesting information was, however, obtained   The number of rodents handled, other than *rattus*, was

1,270 and of these 722 were obtained singly and searched for fleas   The results are shown in Table VII   Bandicoots, as has been found in other places, harbour more fleas than *rattus* and are favoured by *astia* rather than by *cheopis*   The virtual absence of *cheopis* on the other rodents is very striking   The *astia* index is high on gerbils and moderately high on bush rats —

TABLE VII

Other rodents and their fleas

RODENTS			FLEAS			
			cheopis		astia	
Species	Total	Available for flea count	Number	Index	Number	Index
Gunomys kok	281	177	17	0 1	76	0 4
Millardia meltada	238	136	3	0 0	168	1 2
Golunda ellioti	86	75	3	0 0	237	3 2
Leggada booduga	394	220		0 0	44	0 2
Bandicota sp	22	10	31	3 1	171	17 1
Mus urbanus	73	2		0 0		0 0
Funambulus sp	25	25		0 0		0 0
Tatera sp	151	77	5	0 0	641	8 3

In addition to rodents, thirteen musk rats (*Pachyura* sp) were examined and eleven of these had no fleas, while the other two yielded ten *cheopis*   Four mongooses were free of fleas

As regards fleas other than those mentioned *Ctenocephalides felis* var *orientis* is common on cattle, sometimes in enormous numbers, especially on buffalo calves *Ct felis* var *typica* is the usual flea of cats and dogs both of which are numerous and flea-ridden   One example of a naturally plague-infected flea of this sub-species was obtained in a wandering flea test on 10th February, 1933, at a time when there were rat falls in the house   As this species is rarely found on rats despite the prevalence of both, it is probably of no importance in the spread of plague   *Pulex*

irritans has not been found in the valley although it is known to be common on the neighbouring hills   One specimen of *Ceratophyllus nilghiriensis* was obtained from *Millardia meltada*

PLAGUE IN OTHER ANIMALS

Every effort has been made to obtain evidence of natural plague in wild rodents and a considerable number of trapped animals has been examined without result It is notoriously difficult to obtain carcasses even of *rattu* when plague is prevalent, and we have been fortunate to obtain a few examples of plague infection in other rodents   These are tabulated in Table VIII   Bandicoots are occasionally found dead from plague in villages —

TABLE VIII

Natural plague in wild rodents

Date	Rodent	Species	Distance from habitations
17-10-31	Gerbil	*Tatera* sp	800 yards
2-7-32	Mole rat	*Gunomys lol*	400 ,,
22-10-32	Field mouse	*Leggada booduga*	200 ,,
27-10-32	Field mouse	*Leggada booduga*	200 ,,
21-11-33	Mole rat	*Gunomys lol*	800 ,,
27-12-33	Squirrel	*Funambulus* sp	Near

Small numbers of ' other rodents ' have been infected with plague either cutaneously or subcutaneously from time to time   All these died of acute plague   They included 3 *Gunomys lol*, 10 *Millardia meltada*, 12 *Leggada booduga*, 4 *Golunda elliott* and 9 gerbils (*Tatera* sp)

It has been stated by the villagers that monkeys often die when plague is prevalent and that sometimes the monkeys show buboes   No carcass has, however, been available for examination   As monkeys are regarded as sacred animals no attempt has been made to handle them   In some of the villages, such as Theni, monkeys venture freely among the habitations   Fleas have occasionally been found on tame monkeys in the laboratory, but there is a popular belief that wild monkeys do not generally harbour fleas

An unexpected finding was the susceptibility of chameleons to plague   About two dozen were inoculated as they became available from time to time and every one died of acute plague as confirmed by the usual tests   No fleas were ever found on chameleons and no importance is attached to the fact of their susceptibility to plague

In some plague-infected countries wild rodents are of prime importance as reservoirs of plague infection   Thus plague persists in enzootic form among gerbils in South Africa, susliks in South-East Europe, tarabagans in Manchuria and squirrels in California   Generally speaking human plague in these countries depends on the transfer of infection from these wild rodents to house rodents   It has been suggested to us that surely field rodents must play a somewhat similar part in Indian plague   From the evidence now presented it appears that the unimportance of wild rodents in the Cumbum Valley is explained by the fact that they generally harbour few fleas and do not attract the species most closely concerned with plague, *Xenopsylla cheopis*   At the same time they are highly susceptible to plague and occasionally die from natural plague

CLIMATIC CONDITIONS IN RAT BURROWS

The study of this important subject was commenced only towards the end of 1933   A discussion of suitable methods and some preliminary observations have been published by Buxton (1932)   He concludes that standard Stevenson screen readings of temperature and humidity are of little interest to the biologist   That is no doubt true but we have regretted the lack of observations under standard conditions which would allow a comparison of ordinary climatic conditions with those of burrows and with those of other localities

Using a long distance thermograph a few comparisons have been made of the temperature outside and inside rat burrows   During December, nine observations were made for periods of 24 hours   The shade temperature varied from a maximum of 86 5°F to a minimum of 63°F and the daily range was from 21 0 degrees to 12 5 degrees   Meantime the maximum temperature in the burrows was 79 0°F and the minimum 72 0°F while the daily range was from seven degrees to one degree only   The atmosphere in the burrows was, therefore, affected to only a small extent by diurnal variations of outside temperature

A few comparisons of humidity have also been made   The outside humidity figure was calculated from the wet and dry bulb thermometer readings   The humidity in the burrows was measured on samples of air using the miniature dew-point apparatus designed by Messrs Casella (Buxton, 1933)   The saturation deficiency of the air from the burrows varied from 0 11 inch to 0 14 inch while that of the outside air varied from 0 65 to 0 19   The mean for the month, compiled from 8 a m figures only, was 0 13 inch   It is hoped to continue these observations   The discrepancies in readings from the various instruments available are rather disconcerting   One difficulty is that the indicating scale often covers too wide a range so that the divisions are too small to be easily read

TRANSPORT OF RATS AND FLEAS IN GRAIN

The importance of grain traffic in the spread of plague has been emphasized by many authorities   There is, however, very little actual evidence of the transport

of rats and fleas in bags of grain From the Cumbum Valley a daily consignment of about 300 bags of rice passes into Travancore for the tea-estate employees An occasional bag of gram bran chol im or chillies is also carried These are conveyed in bullock carts about a dozen bags per cart For protection from thieves the carts generally proceed in convoys of a dozen or more As the Cumbum Valley has been an endemic centre of plague for some years past it has been assumed that this traffic must be associated with the appearance of plague in Travancore The bullock carts reach the frontier at daybreak having been halted on the steep ghat sometimes for hours at a time Squirrels and field mice are known to visit the carts at the halts These might lead to an interchange of fleas or the introduction of plague among the wild rodents

At Kumil, the carts are stopped to await customs examination Twelve guinea-pigs were allowed to run loose on a dozen bullock carts in series, the animals being moved from cart to cart at half-hour intervals The guinea-pigs were then carefully searched for fleas This experiment was repeated eight times on different days The total catch of fleas was one female *Ct felis*

As regards rodents the off loading of the grain bags has been watched on dozens of occasions and by different observers but rodents are rarely detected Altogether three field mice and one field rat were discovered among the bags No fleas were recovered from them Numerous field mice (*Leggada* sp) have been seen near the carts from time to time

Although it is not denied that plague infection could have been, and in all probability was, conveyed to Travancore by this route, the evidence so far as could be obtained was negative

DISINFESTATION OF GRAINS BY HEAT

The disinfestation of bags of grain by exposure to the tropical sun is now recognized as an unreliable procedure (Pandit, Menon and Iyer, 1933) These authors demonstrated that under such circumstances fleas tend to burrow to the cooler parts of the grain Some observations on grain bags at Kumili may be mentioned in support of these conclusions A long distance recording thermometer was used to ascertain the temperature reached in different parts of bags of rice exposed to bright sunlight on a platform consisting of a foot of fine sand The results showed that the temperature in the bags did not rise to any great extent except near the sacking on the side exposed to the sun An ordinary thermometer, exposed to sunlight and breeze, recorded a temperature of over 90°F for several hours in the middle of the day, reaching as high as 98°F Over the same period a bulb protected from the sun by one layer of gunny and a six inch depth of rice did not show a rise above 95°F A bulb between the gunny and the rice on the side exposed to the sun recorded a temperature of over 120°F for at least two hours and reached as high as 135°F

On one occasion in Cumbum village a bandicoot was found dead of plague in a grain store The fleas on the carcass, ten in number, were removed About 700 bags of grain were removed and placed singly on a cement platform exposed to bright sunlight throughout a whole day in November 1932 The maximum shade

temperature that day was 82°F At night twelve de-flead guinea-pigs were allowed to wander loose among the bags Next day eleven of these were caught and searched for fleas Thirty-six *cheopis* and *astia* fleas were recovered

Hot air chambers have been in use at Kumili for about ten years for the disinfestation of grain in bags This procedure was introduced to take the place of sun-disinfestation on dull days Observations with the long distance thermometer have shown clearly that this method suffers from the same defect as exposure to the heat of the sun

The temperature in the chamber was raised to 160°F in the course of three or four hours, and the chamber was then allowed to cool During this period the temperature in the bags four inches under the gunny reached a maximum of only 90°F Immediately under the gunny it might rise as high as 120°F These observations were made several times and repeated with bran, cholam and cotton as well as rice Cotton heated up more than the other products, the temperature reaching 110°F in the centre of the bags All these observations show that heat disinfestation cannot be relied upon to destroy fleas in grain bags The temperature necessary for the destruction of fleas is 120°F for 45 minutes

An experiment in Cyanogas fumigation

The time-honoured trapping and poison-baiting of rats as anti-plague measures are open to the serious criticism that the fleas are not destroyed at the same time It might be argued that if these measures are employed during epizootic plague they actually release plague-infected fleas and increase the chances of man being infected Methods which destroy both rats and fleas would appear to be preferable

Hydrogen cyanide gas is regarded as the fumigant of choice as far as the lethal effect on rats and fleas is concerned There are a number of proprietary preparations which are capable of liberating hydrogen cyanide gas when exposed to the air One of these is 'Cyanogas' which is said to contain about 50 per cent of calcium cyanide Preliminary experiments with this substance indicated that it could effectively destroy rats and fleas in burrows

Further experiments in Cumbum village appeared to give highly satisfactory results Epidemic plague occurred yearly from 1926 to 1932 In March 1932 three gangs of operators were set to work in different parts of the village and an attempt was made to fumigate every rat burrow in every house The optimists suggested that human plague in the following season would be completely checked It was recognized, however, that an unknown proportion of the rat population live in the thatched roofs of the habitations where they would be unaffected by Cyanogas As regards the fleas, the burrows are probably more important than the roofs The roofs are exposed to the heat of the sun and when hot and dry must be less congenial for adult fleas and less suitable for larval development

Before the end of the year 11,948 burrows in 2,210 houses out of a total of 3,334 houses in the village had been fumigated Some houses had been treated more than once on account of complaints of rat infestation or rumours of rat falls At first all dead rats collected after fumigation were examined for plague infection but this was given up as the numbers became excessive and the results had been consistently negative While burrows were being inspected prior to fumigation, carcasses

Sun disinfestation and hot air chamber, Kumri

have compiled the human plague figures for Cumbum village and a few neighbouring villages in Table IX   It appears that although all these villages had suffered severely from plague, in the course of the seven years, yet in several years one or other of the villages had no human plague cases   Even Cumbum village practically escaped in 1928   Cyanogas was not in use then   It is impossible to deny that Cumbum village might perhaps have escaped in 1932 even if fumigation had not been started   Further observations are, therefore, required before we can assess the exact value of fumigation   So far the results are encouraging —

TABLE IX

Plague cases in Cumbum and some neighbouring villages, 1926–1933

VILLAGES

	Cumbum	Gudalur	Pudupatty	Hanumanthampatty	Chinnamannur	Uthamapalayam	Narayanathevanpatty
Distance from Cumbum, miles —		4	1½	2½	10	6	2
Direction —		S	N	N	N	N	E
Population —	18,844	16,197	3 560	3,368	12,816	9,357	5,967
Plague cases 1926	Nil	Nil	Nil	Nil	320	29	
„ 1927	418	154	198	18	36	169	Nil
„ 1928	5	286	Nil	1	42	81	144
„ 1929	185	Nil	1	Nil	Nil	124	5
„ 1930	80	263	133	129	62	12	Nil
„ 1931	142	42	Nil	Nil	51	101	80
„ 1932	38	408	53	140	55	54	41
„ 1933	10	4	2	168	88	163	Nil

PRACTICAL POINTS IN CYANOGAS FUMIGATION WORK

With intelligent supervision of the work there is no danger to human beings from Cyanogas fumigation   The only symptom observed as a result of the

operations was a severe conjunctivitis in one of the menials who accidentally introduced some of the powder to his eye   The operators did not require masks One opening of a burrow is selected   The nozzle of the Cyanogas pump is introduced for about a foot and the opening is then closed by packing mud round the nozzle   Pumping is commenced and the escape of the smoke-like powder shows the connected burrows   These are promptly closed with mud   According to the extent of the burrow system from six to twelve strokes of the pump are required   Openings from which no smoke appears are treated as separate burrows   The person manipulating the pump should stand on the windward side if he is working in a breeze

Burrows opening outside the house must also be discovered and treated Burrows often pass from one habitation to another and it may be necessary to warn the occupants of neighbouring houses to vacate their premises temporarily and to remove children and domestic animals   A burrow may open on the top of a mud wall in a situation readily overlooked   On one occasion 25 rats toppled from such a burrow and died, while a burrow at ground level was being fumigated

Occupants were warned not to re-occupy their premises for at least three nights after fumigation but an inquiry in 200 houses showed that 5 per cent were actually slept in the same night and 20 per cent on the second night   The risk to life or health appears to be negligible   Complaints of the smell of decomposing rats were usual a few days after fumigation   This could be obviated to some extent by repairing the cracks in the mud used for blocking the burrows   As regards the duration of the lethal effect inside the burrows, experiments on 123 burrows showed that the atmosphere in the burrows might remain lethal to rats for some days On one occasion two rats promptly died when introduced into a burrow which had been fumigated twelve days before

Inquiries have been received regarding the cost of fumigation   The price of the powder varies from Re 1-4-0 to Re 1-12-0 per pound according to the packing One pound of the powder will suffice for the fumigation of about 100 burrows   The pump costs about Rs 25   A gang consists of an intelligent laboratory attendant and two coolies   The gangs have been supervised by plague inspectors who of course have other duties such as inoculation

DISCUSSION

The recrudescence of plague

The usual textbook explanation of the genesis of plague is that the infection persists in a chronic form in the *Rattus norvegicus* population during the plague-free season, and that when the plague season begins the young susceptible *norvegicus* rats contract acute septicæmic plague   Fleas spread the infection to *R rattus* and later on to man (Patton and Evans, 1929)   This explanation may have had some meaning in Bombay where most of the early work of the Plague Research Commission was carried out, but it is most unfortunate that it should have been considered as generally applicable   Nothing is further from the truth   No mention has been made of *Rattus norvegicus* in the Cumbum Valley   This species is not found in India except near the large ports

The life of a starved flea varies enormously, depending on the climatic conditions Bacot and Martin (Plague Research Commission, 1914) kept unfed infected fleas alive for as long as 50 days at a temperature of 50°F to 60°F   Under Bombay hot weather conditions, on the other hand, with the laboratory temperature rarely under 80°F the maximum life of starved infected *cheopis* and *astia* fleas was found to be only seven days (Webster, 1931)   Infected fleas of another species (*Ceratophyllus fasciatus*) were found by Bacot (Plague Research Commission, 1915) to be capable of transmitting after as long as 47 days starvation at a temperature of about 45°F He noted the important fact that such a starved flea may not be capable of infecting at once when fed but may become so after a further period of as much as 20 days In Bombay at room temperature Webster found that starved infected *astia* fleas might be capable of restarting an epizootic in Madras rats after periods up to seven days (Anderson, 1932)   Hirst (1931) states, ' The data in respect of the maximum longevity of starved infected *X cheopis* under tropical conditions are inadequate, but it is safe to say that the female may survive up to one week   In the hills the duration of infectivity would be very much longer '

In the series of experiments now reported, plague-infected fleas, a mixture of *cheopis* and *astia* species, have been starved under reasonably natural conditions They have been found capable of transmitting infection after periods of starvation up to 29 days when the mean room temperature was about 79°F   This study was begun because Lieut -Colonel King had suggested that persistence of infection in the flea was the main agent in the ' carry over

Whether such periods of starvation are usual in nature is unknown   It seems more probable that wild infected fleas obtain at least an occasional meal, in which case the length of life would presumably be longer and the occasional host would sometimes become infected   The wandering flea test has given a surprising number of positive results and it is clear that if the necessity arose, i e , if rodent hosts were really absent, starved fleas might continue capable of infecting for some weeks There is little doubt that in the Cumbum Valley epizootic plague is smouldering all the year round   Numerous examples have been met with of carcasses of rodents recently dead of plague at times when no human plague was evident   In fact it is rare for more than a month to pass without cases of human plague

The facts are entirely in accordance with the findings of the Plague Research Commission   For example, they clearly recognized that plague in scattered villages is more likely to be carried over in large villages, while in small villages infection might die out and remain absent for many months until re-introduced from without (Plague Research Commission, 1910)   This explains why all the villages do not suffer equally from year to year   (*see* Table IX)

An interesting example was found of a village which claimed to be immune to plague   There was no obvious reason why it should be immune and eventually patience was rewarded by the discovery of a plague-infected rodent carcass in that village   This activity on the part of the field workers roused the ire of the inhabitants, but inquiry showed that they had regularly concealed rat falls and had successfully reported some human plague deaths under other diagnoses

The inquiry in the Cumbum Valley was on a very small scale indeed when compared with the epidemiological studies of the Plague Research Commission This seems, however, a suitable opportunity to review certain of their findings

The question of *R norvegicus* h is already been discussed   The reports of the early work of the Commission are somewhat misleading now unless they are read in the light of their later observations   Firstly, the non-pectinate rat fleas of India were believed to be all of one species   We now know that there are three species differing in distribution and importance   This does not greatly concern us here   *Xenopsylla cheopis* is the important flea in the Cumbum Valley   *X astia* can play only a minor part and *X brasiliensis* is absent   Secondly, the mechanism of infection involving Bacot's blocking phenomenon was not understood   Emphasis was, therefore, laid on the total number of rat fleas   Certainly there were often more fleas when plague was prevalent but it would be quite logical to explain that there are more fleas per rat because the fleas are concentrated on the surviving rats   We now know that it is not the total number of fleas, it is not even the number of infected fleas, it is the number of infective fleas that is of prime importance   This depends on climatic conditions   Given the presence of plague in rats and a *cheopis* index of at least one, there will be more infective fleas and, therefore, more plague within a certain range of climatic conditions

SUMMARY

A field study of plague in an endemic focus in South India is reported   Climate, social conditions, rat susceptibility and specific flea population are all favourable to the spread of plague   For a few months in the spring the climatic conditions are unfavourable or borderline   At any other time of the year plague might reach epidemic proportions   The gap between epidemics may be bridged partly by starved infected fleas which have been shown to be capable of transmitting infection after at least 29 days starvation in rat burrows   Rodents recently dead from plague have frequently been found when there were no cases of human plague, and there is considerable evidence that acute septicæmic plague occurs continuously in the rat population   At the same time even human cases of plague occur in most months of the year

The findings are in accord with those of the Plague Research Commission With regard to the Commission's reports emphasis is laid firstly on the fact that *Rattus norvegicus* is not found in the plains of India but only near the ports, and secondly on the necessity of distinguishing *infective* fleas from merely *infected* fleas

Notes are added regarding plague in field rodents, climate in rat burrows, heat disinfestation of grain and Cyanogas fumigation as a plague preventive measure

Permanently to abolish plague infection from the Cumbum Valley by human endeavour can scarcely be regarded as a practicable proposition   There will, however, be less human plague the more the people learn to discourage rats from sharing their habitations   Anything which deprives rats of food and shelter is an anti-plague measure   In the absence of rats, fleas may continue, capable of infecting for 1 month or more

Under existing conditions Cyanogas fumigation is a promising measure   The degree of success following its use will depend on the promptitude with which rat falls are reported

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FURTHER OBSERVATIONS ON INDIAN RELAPSING FEVER

Part I

TYPES OF SPIROCHÆTES FOUND IN EXPERIMENTAL INFECTIONS

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CONTENTS

I   INTRODUCTION

In 1925, in a paper read before the Royal Society of Tropical Medicine and Hygiene, we published the results of our observations on the serology of relapsing fever in Madras (Cunningham, 1925)   The results we had obtained up to that time led us to believe that the spirochæte in the relapse differed serologically from the spirochæte present in the first attack and that these two strains of spirochæte

altcrnated with each other according as the one or the other initiated the primary attack in different animals. At the same time we noted the fact that, while two types of spirochæte sufficed to explain the probable mechanism where the disease was limited to two attacks only (a first attack and one relapse) it was not sufficient to account for the occurrence of more than one relapse in the same animal. Our immunization and protection experiments, and especially the results of our examination of human sera, suggested the possible existence of one or more additional types of spirochæte and we considered that the causation of a second and subsequent relapses in the same animal (a rare occurrence in the Indian type of the disease) called for further investigation.

On our return to India we had the opportunity, between 1925 and 1929, of carrying out further work on this subject which resulted in the discovery and isolation of seven additional antigenic principles, bringing the total number of types isolated from the original Madras strain to nine. These results were exhibited at a meeting of the British Medical Association in Edinburgh in 1927 and, later in the year, at the 7th Congress of the Far Eastern Association of Tropical Medicine held in Calcutta (Cunningham, 1927a). Pressure of other work has, however, prevented our publishing the details of this investigation *in extenso* and, as we consider that such a course may be of value to other workers in the same field, we are now taking the opportunity of doing so.

In the meantime much work on the relationship of various strains of the relapsing fever spirochæte and the spirochætes found in the different febrile attacks has been published by other authors. Brussin (1925, 1926) and Brussin and Rogowa (1927) showed by means of the thrombocytobarin reaction that the ' first attack ' and ' first relapse ' spirochætes in infections of *Sp. duttoni* were serologically distinct but that the spirochætes in subsequent relapses, although they differed from those of the original attack, did not produce specific anti-bodies.

Jakimow (1928, 1929), on the other hand, found, also by means of the adhesion test, that the relapse type of a Berlin strain of *Sp. recurrentis* differed from the ' first attack ' type in about fifty per cent of cases only. Further, that the relapse strain gradually reverted to the original type. In the course of his experiments, however, he identified a third type of spirochæte.

By means of inoculation experiments Gou (1928) showed that the spirochætes in the first and each subsequent relapse in *Sp. duttoni* infections differed from each other. He explained his results by assuming that the spirochætes acquire new immunological properties after each relapse.

Anstowsky and Wainstein (1929, 1929a) and Wainstein (1929) immunized human beings with killed ' first attack ' and ' relapse ' spirochætes respectively and found that protection was afforded against infections with the homologous but not the heterologous strains.* Similar results were obtained with cultures. It was further shown that the various strains preserved their immunological identity on culture.

Using our technique but with splenectomized squirrels, Melency (1928) produced six different relapse strains from a single human strain of *Sp. recurrentis* in North China. These strains retained their specificity during the period of experimentation

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* These results coincide with those we obtained with spirochætal vaccines made from ' first attack ' and ' relapse ' strains in monkeys and squirrels (Cunningham, 1925)

Certain relationships which were found to exist between strains corresponded in principle with our previous findings   Meleney holds, however, that there is a broadening zone of strain relationships which finally causes overlapping in the serological reactions of the later descendants of the original strain

Russell (1931) also noted alternation of types in the louse-borne relapsing fever of the Gold Coast and found a third serological type in certain relapses   It also appeared from tests which she carried out with our Madras sera that her original attack strain (' A ') was related to our relapse strain (' B ')   In a later publication (1933) this author states that she has isolated a fourth type and concludes therefrom that relapsing fever spirochætes have the power of unlimited serological variation

That profound changes can also take place in spirochætes as the result of passage through the brains of infected mice has been shown by Soficff (1930) and Sagel (1930)   Strains which were serologically distinct became undistinguishable from each other after passage and recovery from the brain

Rubinstein and Kaposto (1931), by inoculation of mixed infections of *Sp recurrentis, duttoni* and *marocana*, produced a new but evanescent race of spirochætes which differed in antigenic properties from the three original strains as tested by the adhesion phenomenon   After six passages, however, the strain disappeared and was replaced by the original strain

In contrast with the views quoted above Gray (1929) was unable to demonstrate the presence of anti-bodies either in the human being or in the experimental animal after infection with a laboratory strain of *Sp duttoni* , nor were the spirochætes of the various attacks distinguishable by immunity reactions   These findings receive support from the observations of Plaut and Grabow (1930) who believe that there is little evidence that the termination of each attack is dependent on the production of anti-bodies   They state that the serum has no protective value until after the earlier relapses (up to four) and that a protective action is only then gradually acquired   They claim, therefore, that the disappearance of spirochætes at the end of each febrile attack is still unexplained   On the other hand it is held by many authorities (*see* Hindle, 1931) that the lysis of the spirochæte is a most important factor in the termination of the attack and this view receives recent support from Kritschewski and Singuschina (1931)

It is obvious that there is still considerable diversity of opinion on questions bearing on the mechanism of relapses and immunity in relapsing fever

Much of this want of agreement is, in our opinion, due to variations in the conditions of experiment   In the first place many researches have been carried out with old laboratory strains whose origin and previous history with regard to relapses is quite unknown but whose very survival under laboratory conditions labels them, whatever their present name as belonging to the more resistant tick-borne, rather than the louse-borne varieties which require such frequent passage and are so easily lost   In the second place, the tick-borne variety with its evanescent attacks and relatively feeble blood infection does not lend itself so readily to direct tests for the presence of anti-body as does the more pronounced infection of the louse-borne strain   In fact, it might almost be said that the particular method of experiment depends greatly upon the particular variety of spirochæte being used for the tests ; at least we found this to be the case when we attempted without success to use our technique with certain strains of *Sp duttoni* with which we had kindly

been supplied in this country   The literature shows, we think, that those who have worked with undoubted louse-borne strains are in general agreement regarding the influence of anti-bodies upon the course of the disease and the formation of serological types, whereas opinion is much more at variance amongst those who have dealt with strains of *Sp duttoni*   There is, however, even in the former case, some diversity of opinion as to the stability of the types appearing in relapses, and the whole question calls for further investigation with a view to reconciling, if possible, the various shades of opinion expressed on the subject

II   TYPES OF SPIROCHÆTE FOUND IN EXPERIMENTAL INFECTIONS

Our previous investigation concerned the isolation of two types, the one found in the first attack, the other in the relapse, this being the usual form of infection presented by the louse-borne Indian variety of the disease   The present investigation deals for the most part with the characteristics of the additional types found in the occasional second relapses (third attacks) which occur in experimental animals and the modifications in our views necessitated by their discovery

At the outset, however, it is necessary to define what we consider a relapse for the purposes of this research   The transitory appearance of spirochætes during the first interval has been referred to by certain observers as a phenomenon distinct from a true relapse   This observation must have reference to the disease in the human subject where additional evidence of the relapse is present in the form of a rise of temperature accompanied by a definite clinical picture   In the experimental animal, however, no such relationship exists, so that the presence of spirochætes in the peripheral circulation remains the only criterion of a relapse   For the purposes of the present investigation, therefore, we have taken the reappearance of spirochætes in the blood following a negative finding as the equivalent of a relapse, without reference to the numbers found or the length of time they are present in the blood

Under these conditions a second relapse (i e , third attack in the same animal) was only detected on thirty-three occasions during the whole of the present investigation (i e , about 4 per cent) *   This was the case in spite of the fact that a very large number of infected animals were constantly under observation for the occurrence of relapses   It must be remembered, however, that many of these did not live long enough to exclude the possibility of a second relapse occurring had they survived   It is possible, therefore, that the phenomenon may be more frequent than our figures indicate   Under the most favourable circumstances, however, a third attack in the same animal must be very rare   Only one case was noted in a monkey   The remainder occurred in squirrels

1   *Technique*

(a) *Strains of spirochæte in use* —The strains of spirochæte used in the experiments were derived from human cases suffering from *Sp carteri* infections in different

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* In two series of animals infected with strains derived from separate sources (Madras and the Punjab) the second relapses numbered 21 (5 9 per cent) in the first series of 353 animals and 4 (3 5 per cent) in the second, of 114   All these animals were examined long enough to make a definite pronouncement on this question possible, i e , a minimum of 16 daily examinations after the end of the first relapse, the longest second interval observed in positive cases being 12 days

puts of India   The Madras strain, referred to in our last paper, was the one chiefly used in the present series but three other strains obtained from sporadic cases of the disease in the Punjab (two from Multan, labelled 'P₁' and ' P₂ ' respectively, and one from Hazro) were also studied

The strains and the various types of spirochæte derived from them were kept alive by repeated passage in squirrels (*Sciurus palmaris*)   Monkeys were also used but were reserved for special purposes rather than the routine upkeep of strains

Nicolle and Anderson (1928) advise against the use of wild animals for the maintenance of strains   While agreeing fully with their views as to the great care necessary to avoid fallacies and mixture of strains we consider that the use of wild animals is unavoidable in the type of investigation undertaken by us, for no laboratory could otherwise maintain the supply of animals required for any length of time   In fact we would go further and say that much of the confusion that at present exists on this question is probably due to the restrictions placed on the sub-passage of strains due to an insufficiency of laboratory animals available for the purpose

Our squirrels were at times infected with two other blood parasites, a trypanosome, probably *T lewisi*, in Madras, and *Sp minus*   Contrary to the view expressed by certain authors (Kawamura, 1931 , Velu, Balozet and Zottner, 1931) we did not observe that the presence of these parasites had any effect, provocative or otherwise, on the course of the relapsing fever infections

(b) Methods —The greatest care was taken to prevent mixture of strains by direct infection from animal to animal   Each infected squirrel was caged separately and, as an additional precaution, was marked on inoculation with a distinctive colour for each type of spirochæte so as to avoid any possibility of error through mistakes in labelling   Our method of handling and obtaining blood from our animals has already been described to a great extent (Cunningham, 1927)   Intracardiac puncture, which was originally used in Madras, was discontinued in favour of bleeding from the internal saphenous vein   This method was found equally efficacious if ordinary paraffin oil was applied to the skin before puncture to avoid clotting, and had the additional advantage that fatalities were much reduced   Sub-passages were carried out by intraperitoneal, or, in some cases, subcutaneous inoculation

The terminology employed in our previous paper, while presenting certain advantages, was undoubtedly complicated and has apparently given rise to some difficulty in understanding our conclusions

In order to prevent any misunderstanding in the present investigation the different spirochætal types have been designated by simple letters and little or no attempt has been made to place them according to the number of relapses they have passed through since they arose from the original strain   Thus, the original ' first attack ' spirochætes of the Madras strain have been called ' A ' and the ' relapse ' spirochætes ' B '   All spirochætes from whatever source which gave the same serological reactions as ' A ' spirochætes were known as ' A ' , similarly, all spirochætes giving the reactions of ' B ' spirochætes were called ' B ', and so on   Careful pedigrees of each strain have been kept from the time of isolation so that the number of passages and the relationships of the various types to each other could be

ascertained at any moment   Sera for test purposes were, as a rule, obtained by inoculating monkeys with the appropriate types as it was found that, with spirochætes which had become acclimatized to squirrels, the attacks in the monkey were limited to two or three days only, and, as will be seen later, a more specific serum was thus obtained

Spirochætocidal and agglutinating substances were, as before, used as a measure of the anti-body content of the blood   Our technique for their detection has already been described *   New types of spirochæte were found by testing the organisms causing various relapses with specific sera derived from all the types of spirochæte previously isolated   When no reaction was obtained with any of the type sera, the known types of spirochæte were in their turn tested with a ' first interval ' serum derived from the organism under investigation   If no serological relationships were discovered, the new type was labelled with a distinctive letter and maintained by separate sub-passage, the test serum prepared from it being added to those already in existence

In this way a gradually increasing number of type spirochætes and sera came into existence as the investigation proceeded   These were used as a routine in all tests carried out to prove specificity   Each type spirochæte was preserved by sub-passage into a fresh animal every forty-eight hours, the purity and stability of the various strains being proved by repeated tests with the type serum obtained when the organism was first isolated

In certain cases which relapsed for the second time the organism responsible for the first relapse was not identified at the time   This difficulty was overcome by the routine examination of the serum taken at the onset of the second relapse   the agglutinins present at that time indicated the organisms responsible for the previous attacks

2   *Types of spirochæte isolated*

By these means seven new serological types of spirochæte, in addition to the two primary types already mentioned (types ' A ' and ' B '), were ultimately separated out   These types† became known by the letters ' C ', ' D ', ' E ', ' F ' ' G ', ' H ' and ' I '   Five of these (' D ', ' E ', ' F ', ' G ' and ' H ') were obtained primarily from second relapses ‡   The last one (' I ') was found in a first relapse but has been included here for the sake of convenience

Each type was apparently a distinct antigenic entity, for, in its pure state, it produced a specific anti-body which showed no reaction with any of the other types

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* For the benefit of those who have not seen our previous paper the following summary of our technique is given   the presence of spirochætes in the blood was detected by means of the dark ground illumination   Agglutination tests were carried out by mixing equal quantities of various dilutions of the serum under test with blood containing spirochætes and examining at once under the dark ground for the presence of clumps or spirochætocidal substances   The highest dilution in which any reaction was detected was taken as a measure of the amount of anti-body present   Spirochætes were typed or the anti body content of serum to any particular type of spirochæte was ascertained in this way

† It has been found convenient to refer to the two types ' A ' and ' B ' as ' primary ' types   Those isolated later have been similarly called ' secondary ' types

‡ Type ' C ' was first obtained in a special series of experiments to be described later but also appeared in second relapses in common with the other types of spirochæte

and was itself untouched by any of their specific sera   Chart 1 gives examples of the
infections in which these spirochætes appeared and also the serological tests carried
out in each case to prove their identity   It will be noted that in the first seven cases
(Squirrels Nos 307 to 7056) the results are clear cut   The remainder are examples
of attacks in which more than one type of spirochæte was detected   Further
reference to these will be made later

The anti-body present in the serum of the infected animal at the onset of the
second relapse is shown in the last column   The evidence obtained from these
tests as to the types of organism causing the preceding attacks corroborates those
carried out on the spirochætes present during the actual attacks   It is to be noted,
however, that the relative titre of the agglutinins does not always correspond with
the sequence of the attacks   thus, in the first five examples given (Squirrels Nos
307 to 850) the titre of the agglutinins to the organism causing the first relapse is
much higher than that produced as the result of the preceding first attack, whereas
certain of the other examples given show the reverse effect (Squirrels Nos 4637 and
1733)   A study of complete agglutinin curves shows that high titres are of com-
paratively short duration   the anti-body content of the blood rises very rapidly
after an attack but soon drops to a lower level (*see* Chart 6)

The different types of spirochæte are for the most part merely serological
entities   No differences in morphology or behaviour were seen as far as observation
with the dark-ground illumination was concerned   Actual measurements were not
made but there was no evidence to suggest that this method of investigation would
detect any distinctions between the types

Types 'H' and 'I' were exceptions to the general rules   While resembling the
other types morphologically they produced a peculiar form of attack which in many
cases was unduly prolonged and failed to show the usual picture of an infection
rapidly increasing in intensity and terminating by crisis   A truer conception of
the peculiar features of these two types, however, will be obtained if the serological
tests carried out with them are reviewed at the same time   Their further considera-
tion is, therefore, deferred to a later section

While all the types isolated represented different antigenic principles certain of
them proved to be more definite and tangible than the others   Types 'A', 'B', 'D'
and 'E' fall under the former category   Each of these types was originally isolated
in the pure state and, as will be seen later, retained its distinctive characters almost
indefinitely

The serological characteristics of the remaining strains, while quite definite
when the strain was pure, were not so clear cut and were more liable to change
The 'F' antigen, in particular, exhibited these tendencies   When first isolated
from a relapse it was undoubtedly pure but on sub-passage other elements appeared
and the type soon disappeared   It showed close affinities to the 'B' type, which
usually supplanted it, but it was also found in conjunction with the 'G' type   As
the 'F' type was so unstable comparatively few tests could be carried out with it
Specific 'F' sera were, however, in constant use for identification purposes

The 'G' type also exhibited certain peculiarities   When first isolated its
antigen appeared to be insolubly bound to the 'A' type with the result that a
triangular reaction was obtained when cross-agglutination tests were performed

with the two types the ' A ' type spirochæte reacting with both sera, the ' G ' type with its own serum only, thus —

Spirochæte type	Serum	
	' A '	' G '
' A '	+	+
' G '	−	+

After some time the ' G ' antigen was separated out by means of an anti-' A ' serum which inhibited the growth of the ' A ' elements Once this had been done the ' G ' type became a definite and distinct antigenic principle similar in every way to the other types *

The different spirochætal types were all capable of initiating an infection in experimental animals These infections followed the usual course (with the exception of the ' H ' and ' I ' types) which on occasion included a relapse always caused by a different type of spirochæte

The majority of the tests carried out in the course of the investigation did not include the types ' F ', ' H ' and ' I ' The first of these types was rarely found and maintained a separate existence for a short time only The two latter types were isolated after the greater number of the tests had been completed

III Serological relationships of spirochætal types

Our earlier work envisaged a simple mechanism of attack and defence where the first attack was caused by one type of spirochæte and the relapse by the other each attack being followed by a rise in the anti-body content of the blood

As other types of spirochæte came to light and the tests were extended to include these also, it was gradually found that the blood often contained anti-body to other types which had not, so far as was known, taken any part in the attack or the relapse This point is well illustrated in Table I which shows the results of an examination of the blood of a series of squirrels carried out some time after all signs of the infection had disappeared (i e , periods between 55 and 163 days after the date of infection) None of these squirrels has suffered from more than one relapse and in many there has been only one appearance of spirochætes in the blood Nevertheless, agglutinins of from one to five different types were found in the serum, the majority of cases showing that at least three types of spirochæte were implicated in the completed infection It is of special interest to note, too, that in two out of three instances where no visible infection was noted (Nos 9245 E , 8694 E , 8661 G) agglutinins to the infecting organism were present in the serum These findings showed that the attack and defence mechanism was undoubtedly more intricate than was at first supposed and required further study

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* The original three cornered reaction obtained with this type appears to be similar to that obtained by Meleney (1928) with his strains Va and Vb which bore a similar relationship to his strains II and III

For this 'purpose the course of a series of infections initiated in squirrels and monkeys by the different types of spirochæte was followed in detail

The presence of spirochætes in the peripheral blood was noted daily , the type of spirochæte responsible for the relapse was also ascertained wherever possible, and a complete examination of the serum agglutinins was carried out at suitable intervals so that the agglutinin curve to each could be mapped out in detail   The results of this study have been embodied in a series of charts (Charts 2 to 9) which have been arranged to show the sequence of events in cases suffering from (*a*) a single attack, and (*b*) a primary attack followed by a relapse —

(*a*) *Appearances seen following single attacks* — A study of a large number of agglutination curves following a single appearance of spirochætes in the blood shows that these assume one of four forms   The first of these (Chart 2) shows a simple response to the type of spirochæte causing the infection   the agglutination curve begins to rise towards the end of the attack, especially if clumping of the spirochætes has occurred or, if not, immediately after their disappearance   Agglutinins to the other types of spirochæte are absent

The second form (Chart 3) shows the primary rise initiated by the infecting spirochæte, in this case type ' E '   There is in addition, however, a secondary rise to another type which follows the first and which in this case (Type ' A ') begins sometime after the tenth day 1 *e* , the fourth day after the end of the first attack, so that by the nineteenth day the titre has risen to a 1 in 320 dilution   Agglutinins to a third type may appear at a later period (*see* Chart 5a) so that this chart has the general appearance of two or more curves rising *successively*, the summit of each being lower than the one preceding it

In the third form (Charts 4 and 4a) the usual primary curve to the infecting organism predominates but one or more secondary curves to other types make their appearance at the same time   Chart 4a shows a case with no less than four of these subsidiary rises, the titres in each case gradually decreasing in intensity

The fourth form of curve (Charts 5 and 5a) amounts to a combination of the two preceding forms, where simultaneous rises to two types are associated with successive rises to other types   Chart 5 shows an associated rise to types ' A ' and ' G ' followed by a subsidiary rise to type ' C '   In Chart 5a the primary curve to type ' A ' is followed by two curves to ' B ' and ' C ', rising together and followed in turn by a third curve to type ' E '

Special attention is drawn to the fact that the various appearances noted above are the outcome of a single attack and that in each case the infecting spirochæte, when tested before or in the earlier part of the attack, contained only one antigenic element as far as could be ascertained by the serological tests employed

(*b*) *Appearances seen where there is a primary attack and one relapse* — If attention is concentrated for the present on the reactions which follow the relapse, it can be said that they take the same form as those already described as following the primary attack   Chart 6 shows the formation of a single anti-body to the spirochæte responsible for the relapse (Type ' A ')   Chart 7 shows a relapse giving rise to two curves following each other in succession (Types ' A ' and ' E ')   The third form of curve is seen in Chart 8 where agglutinins to the spirochæte found in the relapse (Type ' C ') and two other subsidiary types (Types ' E ' and ' D ') have appeared simultaneously

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In each of the above examples the agglutinins produced by the primary attack are of course present in addition and these may also conform to any of the forms of curve described so that it is possible to get combinations of any of the three forms as the result of the two attacks   Chart 9 illustrates this point very well   In it the second form of curve follows the primary attack   the third form, the relapse , the final result being the presence of agglutinins to four different types of spirochæte

If the hypothesis is accepted that each spirochætal type or antigen produces its own agglutinin and that the presence of agglutinin is evidence that the type of spirochæte in question has actually invaded the body then it would appear that the disease in its characteristic form consists, not merely of one or two obvious attacks, but of a series of waves of infection of varying intensity caused by different types of spirochæte

In a certain proportion of cases the infection is a simple one confined to one type of spirochæte alone but more commonly the initial attack is followed by others which may or may not become evident as relapses according to the intensity and duration of the infection   The course of events in many cases which exhibit a ' single ' attack is thus very similar to those which show relapses, the difference being only one of degree, in that the attacks are latent in the one case and demonstrable by direct blood examination in the other

If titre of agglutination can be taken as a measure of the intensity of infection it would appear that the spirochætal invasions usually diminish in intensity as the disease advances the possibilities for variation becoming progressively less, until finally the infection dies out completely

IV   Origin of types and time when changes of type take place

This question was left open in our previous paper but one of two alternatives was suggested, (*a*) that the types of spirochæte causing both the primary attack and the relapse were present from the moment of infection, the one developing more slowly than the other so as to produce the relapse, or (*b*) that the disease was due to a single strain which had the power of undergoing a complete serological ' variation ' under the influence of anti-body formation

Certain experiments, originally devised to test the correctness of these hypotheses, have a definite bearing on this question and may, therefore, be conveniently considered here

It was argued that if the types of spirochæte responsible for the primary attack and relapse were present at the time of infection, albeit in vastly different proportions, it should be possible by careful immunization with one type followed by infection with the other, to produce a permanently pure strain which would show no relapses either in the immunized animal or in sub-passages made from it, or, conversely, by immunization followed by infection with the same type to suppress the first attack and permit the development of the relapse   Both of these hypotheses were tested experimentally

Experiment I   Immunization with one type of spirochæte followed by infection with the other —

The experiment was carried out in three stages, (*a*) Immunization, (*b*) Infection, and (*c*) Sub-passage (*see* Table II)

(a) *Immunization* —Both active and passive immunization were utilized For the former, spirochætal vaccines were made, from both the ' A ' and ' B ' types of spirochæte used for the experiment, by the same methods as those used in former immunization experiments carried out by us   One of the vaccines (say type ' A ') was given in appropriate doses to each member of a batch of squirrels and the agglutination titre of their sera tested to discover the degree of immunity obtained

(b) *Infection* —When the immunity was considered satisfactory an infecting dose of the other type of spirochæte (in this case type ' B ') was given   For the first three days of the infection each squirrel was also given a daily dose of a high titre serum made from the same spirochætal type as that used for the vaccine (in this case the ' A ' type) so as to ensure, as far as possible, the development of a pure 'B' type spirochæte by the destruction of all the ' A ' elements

(c) *Sub-passage* —After the third day of infection the spirochæte was sub-passaged into a series of fresh squirrels so as to remove it from the influence of all anti-body and permit of its development under normal conditions   Similar numbers of unprotected squirrels were infected and sub-passaged at the same time to serve as controls   The titre of the squirrel's sera and the purity of the spirochætes used for infection were tested at suitable intervals throughout the experiment

Two separate series of squirrels were dealt with, the one immunized against the ' A ' type and infected with the ' B ' type   the other immunized with the ' B ' type and infected with the ' A ' type   The results of the experiment are given in the table (Table II)

In the control series and sub-passages originating from them, seven and five animals respectively died ' early ' (i e , before a relapse had time to develop)   In the animals which survived for a sufficiently long time the proportion of squirrels showing relapses was practically the same in each series   8 animals out of 11 (3 animals did not relapse) and 9 animals out of 13 (4 animals did not relapse)   In the immunized series, on the other hand, the deterrent effect of immunization on the occurrence of relapses was quite definite   The more frequent manipulations were responsible for a higher proportion of early deaths, but in the nine survivors only three relapses occurred   Two of these animals were insufficiently immunized, for both had an evanescent relapse in which the type of spirochæte used for immunization was found   The relapse in the third case was caused by a third type, ' C ' against which no immunity had been induced

In the animals *sub-passaged* from the immunized series, however, nine relapses were observed out of eleven infections (two animals showed no relapse)   It was obvious, therefore, that the immunization of the primary series had no effect on the subsequent development of the spirochæte, which, it was thought, might have been permanently eradicated by this means

Experiment II   Immunization and subsequent infection with the same type of spirochæte —

This experiment was carried out in precisely the same way as the previous one with the exception that the same type of spirochæte was used for immunization and infection   Four squirrels were immunized and then infected with the ' A ' type spirochæte and four non-immunized animals were used as controls (Table III)

In three out of the four immunized animals the primary attack was completely aborted without the appearance of a relapse   In the fourth squirrel a relapse lasting at least four days appeared without any evidence of a primary attack   The spirochæte in this case belonged to the ' C ' type [*]

These two experiments taken together give some valuable information as to the origin of types of spirochæte secondary to the infecting type   In the first place our first hypothesis cannot be accepted in so far as it demands the presence of two or more immutable types of spirochæte throughout the course of the disease, for it was found impossible to produce a permanently pure strain by the elimination of the ' relapse ' type, although the methods employed were sufficient to destroy much larger numbers of the same type of spirochæte when used for initial infections   On the other hand the second experiment shows that in many cases the secondary strains are not present at the moment of infection and so must develop later in the course of the disease, as both attack and relapse were aborted in three out of the four cases infected [†]   The fourth case, in which a relapse occurred, can only be explained on the supposition that the variant was present in the presumably pure strain when used for infection, or that certain individuals were able to change their antigenic properties so rapidly that they were able to resist the destructive action of the anti-body

These experiments give us the conception of an organism composed, not so much of pre-existing antigenic elements, as of potential variants called into being in response to various factors of which anti-body formation is possibly one   By this means the organism is repeatedly able to avoid destruction and to produce the characteristic relapsing features of the disease

The change of type is obviously a gradual process and occurs at different times during the course of the disease   The variants may be present in the inoculum from an apparently pure strain but more commonly they appear to be formed as the disease develops

Evidence of the presence of additional types is often found at the end of the first attack initiated by a presumably pure strain   Thus, free-swimming spirochætes in small numbers may often be seen accompanying or following the culmination of

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[*] The experimental suppression of the first attack has previously been reported by Novy and Knapp (1906) and other authors but we have also found it to occur occasionally in the ordinary process of sub passage

The following examples of this can be given —

```
                                        ' B '
Sq  No  2957 G   — — — — — — — — — + + + — — — — — — — —
                                      ' A '
Sq  No  3615 B   — — — — — — — — — — + + + + — — — — — — — — — — etc
                              ' AG
Sq  No  3005 C   — — — — — — + + + + + + died
                             ' A
Sq  No  11282 B  — — — — — — — + + + + — — — — — etc
```

Note —Each + or — sign denotes a daily examination

Meleney (1928) has also reported its occurrence   It would appear, therefore, that conditions similar to those obtaining in the above experiment occasionally appear in ordinary infections

[†] Further evidence bearing on the same subject is afforded by the form of agglutination curve following inoculations of N A B in relapsing fever infections

If the curative dose is given early in the infection agglutinins are present to the infecting type of spirochæte only proving that other types are a later development   This question will be dealt with in another communication

an attack in which the majority of the spirochætes have been clumped and have disappeared (*see* Text-figure and Table IV)   In most cases these spirochætes disappear after 24 to 48 hours but they may increase in number and in one example given (No 5889 G) were proved to be a different type to that originally used for infection An extreme case of this kind is also shown (No 3506 C) where three successive waves of infection followed immediately one on the other in the course of a single attack, each wave being brought to an end by clumping of the spirochætes   In such cases it would appear that the new types begin to develop before the end of the preceding attack so that the successive infections are, as it were, superimposed one upon the other (*see* Table IV)

free
spirochætes
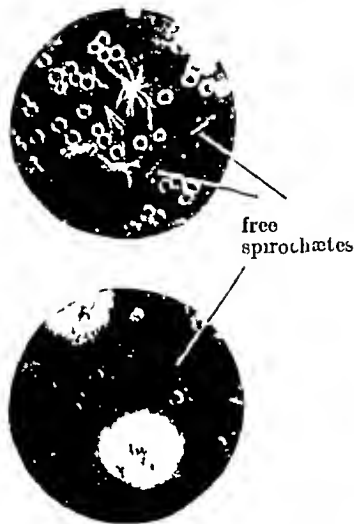
TEXT FIGURE

Dark ground preparations of spirochætes
clumped with specific type serum showing
spirochætes unaffected by the serum

The agglutinin curves already studied (Charts 2 to 9) and the presence of spirochætes themselves in relapses afford abundant evidence of the development of new types later in the course of the disease   It can be concluded, therefore, that secondary types may take form at practically any period of the disease, their presence and numbers depending upon the factors which govern the ebb and flow of attack and defence

V   MIXED TYPES

Cases occur in which two, and more rarely three, types of spirochæte appear in varying proportions in the same infection   They are found under varying conditions in both relapses and primary attacks and the sub-passages following from them Their detection followed the observation that, in such cases no single type serum produced agglutination of all the spirochætes present but that a suitable combination of the sera resulted in a complete reaction   The number of spirochætes affected by

each serum varied   In some cases the types were present in approximately equal numbers   More frequently, however, one type predominated over the other to the extent of three-quarters of the total number or even more   An example in which the types ' G ' and ' A ' were found in combination may be given —

Test sera	Dilutions				
	$\frac{1}{10}$	$\frac{1}{320}$	$\frac{1}{640}$	$\frac{1}{1,280}$	$\frac{1}{2,560}$
' G '	$+\frac{3}{4}$	$+\frac{1}{2}$	$+\frac{1}{4}$	—	—
' A '	$+\frac{3}{4}$	$+\frac{1}{2}$	$+\frac{1}{4}$	$+\frac{1}{4}$	—
' G ' + A '	+	+	+	$+\frac{1}{4}$	—

Note —The fractions denote the approximate proportion of the spirochætes agglutinated, the remainder remaining unaffected by the particular serum in use

In this particular case the titre for the two specific sera used was —

' G ' serum $\frac{1}{640}$

' A ' serum $\frac{1}{1,280}$

Both sera agglutinated the spirochætes in the proportions each type was present up to the limit of the titre of the respective sera   The reaction was complete with the mixture up to the final titre of the weaker serum (' G ')   The next dilution (1/1,280), containing only ' A ' anti-bodies, reacted with the ' A ' elements alone, i e , one-fourth of the total number of organisms present

These mixed strains could be maintained by sub-passage at 24 to 48 hours after infection for a considerable number of sub-passages but the proportion of the two types varied, sometimes one strain being predominant, sometimes the other   A more detailed reference will be made to the factors governing these variations when the stability of spirochætal types is discussed

Table V shows the combinations which occurred in 89 instances of 'mixed type' infection   It shows that any type can combine to form a mixed infection with any of the remaining types (with the possible exceptions of ' D ' and ' F ') provided that the conditions which permit of the presence of both types in the blood are fulfilled   The most common combinations seen were ' A ' and ' G ', and ' B ' and ' C '   The figures cannot, however, be taken as an absolute measure of the frequency of these combinations for the conditions under which they originated are not altogether comparable, i e , the different types have not been passed through the same number of sub-passages so that the various combinations have not had the same opportunity of appearing   They serve, however, as an indication of the commoner varieties of mixed infections

VI FREQUENCY OF APPEARANCE AND ULTIMATE FATE OF TYPES

In our previous investigation in which only two complementary types, 'A' and 'B', were discovered, this question did not arise   With the isolation of so many additional antigenic principles, however, the numerous possibilities of variation require consideration

Passage of the organism from animal to animal can occur naturally during the first attack or any subsequent relapse   Each new infection, whether originally derived from a primary attack or relapse, is itself capable of producing a relapse which on sub-passage may once more originate a fresh series of first attacks and relapses   This process can be continued indefinitely, as it were, in a series of steps, or 'succession' of relapses with a change of type each time a relapse appears (*see* Charts 10 to 18)   In this way a large number of different types may arise as the epidemic develops unless some conservative process is brought into operation to check the tendency towards continuous variation   Our early work, admittedly carried out with a limited number of animals, suggested the possibility that some such check did in fact exist

Melency (1928), if we understand him aright, however, pictured the creation of an ever increasing number of types as the infection progressed   His conclusions were based, however, on a small number of tests performed on splenectomized animals in which the disease is admittedly more severe and the change of type probably less under control   It is questionable, therefore, whether his results are wholly applicable to the disease as it exists under natural conditions   The whole question, in our opinion, merits further examination and we make no apology for presenting our results in some detail as the subject is one of the most important problems in the ætiology of the disease

In dealing with this question we have followed three different lines of investigation—

(1) The frequency with which different types have appeared in relapses following infections initiated by each type of spirochæte and their possible relationships

(2) The types of spirochæte found in relapses arising from the main stem of three separate human strains

(3) The ultimate fate of the spirochætal types which originally appeared in second relapses as judged by the types which appear in a ' succession ' of relapses arising from them

(1) *Frequency of appearance of different types and their relationships* —Table VI shows the distribution of the types of spirochæte appearing in 506 first relapses These figures have been collected from attacks which have occurred subsequent to the isolation of the 'G' type so that pure lines of all the types found previously were in existence, although not necessarily in equal numbers   The peculiar types 'H' and 'I' were not discovered until much later and so cannot be considered on the same footing   The table shows that the types 'A' and 'B' which were first isolated appeared far more frequently than any of the others, no matter what type initiated the attack   Apart from this outstanding fact it would appear that practically any type can, on occasion, follow any other, although certain combinations are undoubtedly unusual (e g , type 'G' following type 'A', etc)

Types ' A ' and ' B ' are definitely complementary to each other. The other types take up, as it were, an intermediate position in that either the ' A ' or the ' B ' type can follow them in a relapse as occasion demands. It might also be inferred that types ' C ', ' D ' and ' E ' were more closely allied to the ' B ' than the ' A ' type from the much higher proportion of ' A ' relapses which follow them, while on the same grounds the ' G ' type might claim to be more akin to the ' A ' type. Apart from these indications, however, it is impossible to establish a more precise relationship between the different types.

The ' F ' type was peculiar in that it could not be kept as a separate entity for any length of time and, therefore, could not be studied to the same extent. It appeared to be closely connected with the ' B ' type which generally superseded it even in the primary attacks. The ' mixed types ' call for no special mention here apart from the fact that either the ' A ' or the ' B ' element was almost always the dominant factor in the combination.

The next table (Table VII) shows the types appearing in the second relapses in the 19 cases in which the spirochætes responsible for the two previous attacks were definitely identified. Ten of the 19 cases showed the ' A ' and ' B ' types in the first two attacks. In five of these ' C ' was responsible for the second relapse. The remaining second relapses were caused by various other types or combinations which occurred too infrequently to have any special significance.

Certain general principles which govern the appearance of these spirochætal types can be deduced from the facts which have just been brought forward.

In the first place it can be laid down as axiomatic that spirochætal types responsible for relapses always differ from those found in the attacks which have preceded them. In other words the previous appearance of any particular type in an infection debars it from any further participation in the disease in the same animal. This principle, which emerged from our early work so far as the two primary types ' A ' and ' B ' were concerned, undoubtedly applies to the other types which have been isolated more recently. The two primary types were also considered complementary to each other, a term which was synonymous with the principle enunciated above as long as the two types were involved alone, but does not necessarily follow where other types had to be considered. The additional evidence which has just been brought forward merely strengthens the view previously held with regard to these two basic types. Meleney holds that the remaining types can also be divided into associated groups which appear in alternate relapses and which apparently can only follow certain types. We have been unable to substantiate this view, for, although certain of these secondary types appear to be more closely related to one or other of the primary types than the others, there is no clear cut distinction between them and they all appear to hold a more or less intermediate position which enables them to follow any type when necessary. This peculiar attribute, as will be seen later, undoubtedly affects their stability as independent antigenic elements.

(2) *Relapses arising from the main stem of three separate human strains.*—The three strains ' P_1,' ' P_2,' and ' Hazro ' were obtained from three sporadic cases of the disease occurring in the Multan and Hazro districts in the Punjab. The case labelled ' P_1 ' was found to be due to the ' A ' type ' P_2 ' to the ' B ' type and ' Hazro ' to the ' C ' type of spirochæte.

The ' P_1 ' strain (Chart 10) was carried on for 302 generations in the direct line and throughout the whole of its course remained true to type   The majority of animals did not survive long enough to eliminate the possibility of a relapse occurring, but relapses appeared in 55 instances in the remaining 86 animals   The type of spirochæte present was determined in 16 of these and proved to belong to the ' B ' type, some ' C ' elements being present in three cases (Nos 214, 215 and 243)

The ' P_2 ' strain (Chart 11) was kept alive for 300 direct sub-passages and remained a ' B ' type during the whole period of its existence   Once more the majority of animals died before the possibility of a relapse could be excluded, but of the 90 animals remaining, 54 showed relapses and 34 of these were tested for the type of spirochæte present   The distribution of the types found in these cases was as follows —

' A ' type in 23 cases (some ' G ' elements also present in 3 cases)
' C '   „   „   6
' D '   „   „   1   ,
' G '   „   „   3   „

The ' H410 ' strain (Chart 12), found to be a ' C ' type on isolation, was passed through 88 direct passages   At the 39th passage it changed type to ' A ' during the first attack and continued as an ' A ' until the strain was allowed to die out   In this case 43 animals died too soon to show a relapse while 25 relapses appeared in the remaining 45 animals (12 while the strain remained a ' C ' type and 13 after it had changed to ' A ')   The type of spirochæte was determined in 18 cases as follows —

Before change of type	After change of type
' A type in 4 cases	Nil
' B ' „ „ 3 „	In 9 cases
' D , 1 case	„ 1 case

The results obtained with this strain are of interest from several points of view   In the first place there is a change of type from ' C ' to ' A ' occurring without the intervention of a relapse   A study of the types of spirochætes appearing in the relapses shows that the ' C ' type when first isolated inclined definitely towards the ' B ' side (the majority of the relapses were due to ' A ' spirochætes)   This condition of affairs gradually changed with sub-passage, as the ' A ' elements gradually gained a preponderance until the ' C ' spirochætes disappeared altogether and actually appeared later in the relapse in combination with ' B ' (passage 67)   Once the type ' A ' spirochætes became established as the cause of the primary attack the ' B ' type appeared in the relapse in almost every instance

Considering as a whole the results obtained with these three strains it is obvious that the ' A ' and ' B ' spirochætes predominate as the causes of the relapses in all three strains   This is perhaps to be expected in the first two instances in which

these two types were responsible for the primary attacks   It is of some significance, however, that the same sequence should follow an infection with the ' C ' type of spirochæte and points to a definite trend towards the re-establishment of the two main types ' A ' and ' B '

The other types seem to appear more frequently in the earlier relapses and infections arising from sub-infections originating from them   This may be due in some part, at any rate, to the process of acclimatization of the spirochæte to another species of animal for the infections immediately following the transfer from the human being are frequently atypical and difficult to maintain

(3) *The ultimate fate of spirochætes which have appeared in second relapses as judged from the types appearing in a ' succession ' of relapses arising from them.*—Charts 13 to 18 give examples of the results which follow the sub-passage of each of the ' second relapse ' types, ' C ', ' D ', ' E ', ' F ' and ' G ', through a series of relapses   These, together with the three preceding charts (Charts 10, 11 and 12), illustrate the course of infections initiated by all the types in our possession with the exception of the last two, ' H ' and ' I ', and must, therefore, give a fairly complete picture of what must occur under natural conditions   The organisms have been carried through from two to four *series* of relapses, the first series being reckoned as the one arising directly from the parent stem of the ' second relapse', the second series consisting of relapses arising in sub-passages from the first series and so on

These charts speak for themselves and do not require detailed description a short reference to the salient features of each will, therefore, suffice—

Chart 13, Sq No 396 B   2nd relapse, type ' C '

The ' C ' organism when it first appeared was apparently nearer the ' A ' type as a ' B ' spirochæte appeared at first in its relapse in the first series   On sub-passage, however, it gradually approached the ' B ' type as the later relapses in the same series were due to the ' A ' type   In series II and III the ' A ' and ' B ' types alternated with each other with only one exception (No 411 in the second series which relapsed to a ' C ')

Chart 14, Sq No 392 B   2nd relapse, type ' D '

Here again the earlier sub-passages were followed by ' B ' relapses but the ' B ' elements appear to have influenced the primary attacks (*see* Nos 405 and 422) and the ' A ' type then appeared in the later relapses in the first series   The relapses in the next series were due to either the ' A ' or ' B ' types

Chart 15, Sq No 391 A   2nd relapse, type ' E '

This strain was followed for over 300 direct sub-passages and exhibits several points of interest   The relapses in the first series were more variable than in the two preceding examples and varied between the types ' A ', ' B ', ' D ' and ' G ' with the majority due to type ' A '   At sub-passage No 579 E two parallel series of sub-passages were carried on, one of which favoured ' A ' and ' B ' in its relapses, the other ' D '   In the later series the ' A ' and the ' B ' types again preponderate but the other types ' E ', ' D ', ' C ' and ' G ' make an occasional appearance showing that complete stability had not yet been reached when the examination ceased

Chart 16, Sq No 407 C   2nd relapse, type ' F '

Reference has already been made to the instability of this type   When first isolated it contained ' D ' and also ' E ' elements but these gradually disappeared on sub-passage   The types of spirochæte in the different relapse series vary somewhat ' B ' is the most common type in the first series   Even where other types have been found, however, there is an undoubted tendency for relapses from these to exhibit the primary types ' A ' and ' B '

Chart 17, Sq No 417 A   2nd relapse type ' G '

The relapses from the main stem in this case do not show the same uniformity in either the first or second series   It is of interest to note, however, that the relapses occurring in the long chain of sub-passages made from the ' D ' type in series II (No 429) are due for the most part to the types ' A ' and ' B

Finally, the results obtained in a number of other strains arising from second relapses have been summarized in Chart 18   In these cases also the ' A ' and ' B ' types predominate in the relapses arising directly from the infections caused by these ' second relapse ' organisms   to be precise, in 11 out of 13 instances

The experimental data which have been produced are, we think, sufficient to show that the broad principle underlying these spirochætal types, either in relapses arising directly from the parent stem or in a succession of relapses carried through several series, consists of a definite and persistent attempt to revert to the primary types ' A ' and ' B ' whenever conditions become favourable   There is certainly some variation in the rate at which this process takes place but the whole trend of the experiments points in the same direction   Evidence of unrestricted variation of type is certainly not forthcoming   We do not mean to suggest, however, that the nine antigenic components we have isolated exhaust the possibilities for variation which exist in relapsing fever spirochætes   On the contrary, we hold that this process is a vital one and, therefore, capable of infinite variety given the necessary conditions, but we consider that the constant drift back towards the basic types acts as a powerful check on what would otherwise result in an unrestrained multiplicity of types with complete loss of specificity

The fact that the two basic types have been isolated in areas so far apart as the Madras Presidency and the Punjab is strong evidence in favour of this hypothesis

VII   STUDY OF TYPES ' H ' AND ' I '

These two types, which were isolated towards the end of the investigation, showed certain departures from the ' normal ' which call for special consideration

Morphologically, they showed no difference from the other types either under the dark-ground illumination or in stained specimens   A large proportion of the attacks produced by them, however, instead of developing along the usual lines seemed to hang fire owing to the apparent inability of the spirochætes to develop in any numbers   The attacks thus became much prolonged, the organism persisting in the blood for comparatively long periods of time   Certain of these attacks lasted as long as nineteen days, while a large proportion of them exceeded twelve days in length (Table VIII)

Type ' G ' when first isolated had close affinities to type ' A ', showing a triangular reaction in cross agglutination tests until the two antigens were finally separated by experimental means

4   A study of the agglutinin curves following infections initiated by the various types showed that these conformed to one (or a combination) of four forms following either the first attack or the relapse —

(*a*) A simple response to the type of spirochæte causing the infection, or, in the case of the relapse, the relapse

(*b*) A series of one or more subsidiary rises to other types following the main curve *in succession*, the summit of each succeeding curve being lower than its predecessor

(*c*) One or more subsidiary curves to other types arising simultaneously with the main response to the type causing the infection (or the relapse)

(*d*) A combination of the two preceding forms (*b*) and (*c*) in the same infection or relapse

These different forms of curves or combinations of them could follow the first attack or relapse with the result that the serum at the close of the infection might contain agglutinins to from one to five different types even in cases without obvious relapses   The disease would, therefore, appear to consist in most cases of a succession of invasions by different types which vary in intensity and duration ; the essential difference between cases which exhibit ' single ' attacks and those showing ' relapses ' being only one of degree in that the later attacks are ' latent ' in the one case and demonstrable by blood examination in the other

5   Experiments carried out to determine the origin of the various types give the conception of an organism composed not so much of pre-existing antigenic elements as of potential variants   Change of type is presumably a gradual process and occurs at different times during the disease   The variants may be present in an apparently pure strain on inoculation but more commonly appear to be formed as the disease develops   Evidence of the presence of such variants is sometimes to be found at the end of the first attack

6   Infections in which more than one type of spirochæte is present are to be found on occasions   Such ' mixed ' strains can be maintained for considerable periods by sub-passage but the proportion in which the two types are present varies from time to time   With the possible exceptions of types ' D ' and ' F ' any one type can combine with any other   ' A ' and ' G ', and ' B ' and ' C ' were the most common combinations found

7   The frequency and the ultimate fate of the various types of spirochæte have been investigated in various ways

A   A study of the spirochætes appearing in 506 first relapses arising from infections initiated by the various types shows that—

(*a*) The appearance of any particular type in an infection debars it from any further participation in the disease in the same animal   This principle applies to all the types isolated

(*b*) The primary types ' A ' and ' B ' are definitely complementary to each other   The other types take an intermediate position and although

certain of the secondary types appear more closely related to one or other of the primary types ('C', 'D' and 'E' to 'B' and 'G' to 'A') there is no clear cut distinction between them nor can they be divided into associated groups

B   The same general principles with regard to the appearance of types apply to second relapses also   Ten out of 19 cases showed the 'A' and 'B' types in the first two attacks   In five of these type 'C' was present in the second relapse In the remainder other types or combinations of types appeared

C   An examination of a series of first relapses derived from the main stem of three strains obtained from different human sources (Punjab 1, due to 'A' type, Punjab 2, due to 'B' type, and Hazro, due to 'C' type) shows that the majority were due to types 'A' and 'B' and comparatively few due to other types

D   A large number of the strains appearing originally in second relapses have been followed through a ' succession ' of relapses (*see* page 119) with a view to determining the ultimate fate of such types under approximately natural conditions The experimental data produced show that the broad principle underlying the appearance of these types consists of a definite and persistent attempt to revert to the primary types 'A' and 'B' whenever conditions become favourable   Evidence of unrestricted variation of type is not forthcoming   On the other hand no claim is made for the limitation of the possible antigenic components to those isolated up to the present   The constant drift back towards the basic types, however, must act as a powerful check on unrestrained multiplicity of type which would otherwise result in complete loss of specificity

8   Two types, 'H' and 'I', which exhibited certain peculiarities, were isolated towards the end of the investigation   The attacks initiated by these types were much prolonged (up to 19 or 20 days) and were characterized by the apparent inability of the spirochætes to develop in any numbers   The types were true serological entities and, while remaining consistently true to type at the beginning of the attack, either remained pure throughout the whole attack or changed to another type about the eleventh or subsequent days   It is suggested that the phenomenon may represent a form of resting stage occurring in the peripheral circulation similar to that exhibited by spirochætes when they persist in the internal organs

9   The primary types 'A' and 'B' and certain of the others ('D', 'E' and 'G') are remarkably stable on sub-passage as long as the transfers are carried out sufficiently early (24 to 48 hours after infection)   Other types show a greater tendency to change type after a certain number of generations have passed

10   Change of type without the intervention of a relapse has also been observed on *rare* occasions   When it does happen the course of events is as follows   during the routine upkeep of strains the invading type gradually appears in the first attack and increases in numbers in successive sub-passages until the original type is completely replaced   The change may occur rapidly in the course of a few sub-passages or very gradually requiring twenty or more sub-passages for completion   These changes occurred chiefly in the case of the secondary types and were mainly in the direction of the primary types   Types 'C', 'F' and 'G' were those most frequently involved

TABLE II

Giving the results of an experiment designed to produce a purified type of spirochæte by immunizing against one type and infecting with another type

Series	Experiment number	Number of squirrels	Type of spirochæte used for — Immunization	Type of spirochæte used for — Infection	IMMUNIZED SERIES — Died early	Number or squirrels Infected, Relapse present — Number	Relapse present — Type of spirochæte	No relapse	Not infected	SUB PASSAGES FROM IMMUNIZED SERIES — Number of squirrels	Died early	Number or squirrels Infected, Relapse present — Number	Relapse present — Type of spirochæte	No relapse	Not infected
Experiment	1	10*	B	A	5	2	BC	2	0	9	2	4	?BBB	2	1
	2	10†	A	B	4	1	?	4‡	0	9	2	5	AAAAC	0	2‡
TOTALS		20			9	3		6	0	18	4	9		2	3
Control	1	10*		A	4	4	BBBB	1	0	9	2	5	?BBBB	2	0
	2			B	3	4	AAA?	2	0	9	3	4	?AAA	2	0
TOTALS		20			7	8		3	0	18	5	9		4	0

*1 died before infection developed
†1 died before immunization complete
‡2 became negative after third dose of serum, therefore sub passage not infected
§1 escaped before infection developed

TABLE III

Giving the results of an experiment designed to show whether more than one
type of spirochæte is present in a presumably pure strain by means
of immunization and subsequent infection with the same
type of spirochæte

Series	Number of squirrels	Type of spirochæte used for		Number of squirrels				
		Immunization	Infection	First attack	Relapse present		No relapse	Not infected
					Number	Type of spirochæte		
Experiment	4	A	A	0	1	C	0	3
Control	4		A	4	4	ᵃBBC	0	0

Table IV

Giving examples of infection showing a continuation of the attack after clumping of the spirochætes has occurred

Squirrel number	Spirochæte type	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	Result
												Days of infection										
2231	B	+[15]	⧻	+++	⧻	+++	⧻	⧻ C	+[8]	—	—	—	+[3]	+	+[10]	+[15]	—	—	—	—	—	Died
2503	D	⧻[2]	+[3]	⧻	⧻	⧻	⧻ C	+[6]	—	—	—	—	—	—	—	—	—	—	—	—	—	
371	A	+[4]	+[15]	⧻	⧻	⧺	⧻	⧻ C	+[4]	—	—	—	—	+[1]	+[30]	+[19]	⧻ C	⧻	⧻ C	+[9]	—	Died
3506	C	+[1]	+[2]	+	+[20]	⧻	⧻ C	+[5]	+[1]	+[5]	+[20]	⧻ C	+[7]	+[8]	+	⧺	C[11]	—	—	—	—	Died
5889	G	+[1]	+[20]	+[1]	+[20]	⧺	⧻ C	⧻ C	+[10]	⧻												

A +		A —
B —		B —
C —	Tests	C —
D —		D —
E —		E —
G —		G +

The signs and numerals have the same significance as those in the charts (*see* note to Charts 2 to 9, page 141)

Table V

Showing the combination of spirochæte types which occurred in 89 cases of infection with 'mixed' strains

Types	Number of times the combination occurred								Number of times each type was found in combination
	A	B	C	D	E	F	G	Totals	
A		10	6		3		19	38	38
B			17	3	2	5	7	34	44
C					1	1	1	3	26
D					8		1	9	12
E							2	2	16
F							3	3	9
G									33
Totals								89	178

TABLE VI

Showing the distribution of the type of spirochæte appearing in the first relapse in 506 animals arranged according to the type of spirochæte responsible for the first attack

Type of spirochæte in first attack	TYPE OF SPIROCHÆTE APPEARING IN THE RELAPSE — Number of times each type was found																		TOTALS
	A	B	C	D	E	F	G	H	I	AB	AC	AE	AG	BC	BD	BF	BG	EG	
A		99	12	4	3	1	1		1					4					124
B	67		10	9	2		10			3			4						105
C	37	14		1			1			5	2		3						63
D	48	20	1	14			1			3		1	2	1	1	1	1		78
E	18	7	2	3		1	1			1									44
F		4	1		1														6
G	2	30	15	1	1	4					2			7					64
H																		2	2
I																			2
AB																			
AC		4																	4
AG		8	2																10
BC																			1
BF	2																		2
FG		1																	1
TOTALS	174	187	42	32	8	5	16		2	9	4	1	9	12	1	1	1	2	506

TABLE VII

Showing the distribution of the type of spirochate appearing in the second relapse in 19 animals arranged according to the types of spirochate responsible for the first attack and first relapse

Type of spirochæte in		TYPE OF SPIROCHÆTF APPEARING IN THE SECOND RELAPSE												TOTALS
		Number of times each type was found												
first attack	first relapse	A	B	C	D	E	F	G	H	I	BG	CA	CG	FG	
A	B			2		1		1	1						5
B	A			3	1	1									5
B	E											1			1
BC	A													2	2
C	A						1								1
D	A										1		1		2
E	A		1												1
E	B	1													1
H	C				1										1
TOTALS		1	1	5	2	2	1	1	1		1	1	1	2	19

TABLE VIII

Giving examples of (1) 'standard' attacks and relapses in squirrels for comparison with (2) 'single' attacks and (3) attacks with 'relapses' caused by the 'H' and 'I' types of spirochæte in squirrels

Squirrel number	Spirochæte type	Days of infection
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20

I 'Standard' attacks

Squirrel number	Spirochæte type	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
446	A	5	30		30	+H	‖c														
385	B	1	6	6	12	20	+c					(8)	1	8	(12)						
5838	D	3	30	++	H	+‖	‖c	‖c					(25)	5	+H	+H	‖c				
435	E	4	10	20	+H						(1)	(16)	(20)								

II Types 'H' and 'I' 'Single' attacks

Squirrel number	Spirochæte type	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
4882	H	1	6	(20)	(15)	(15)	(5)	(5)	2	2	1	1	2	1	(10)	(5)	(2)	(1)			
4947	H	1	2 (·)		(20)	(15)	(10)	(1)	(5)	(20)	(30)	1	2	1	1						
	H	(15)		(6)	1 (8)	1 (25)	2	10	4	4			(1)	(2)	(1)	(1)	(5)	(1)			
5	H		(20)		2	(5)	2		(10)		(30)	(10)	(10)	(10)	3 (2)		(10)	(1)	(1)	(5)	
5032	H		(2)		(15)	(12)	(8)	(4)	(8)	1	2	3	1	2	(2)	(10)	(5)	(2)	(10)	(1)	(1)
9013	I	(25)	6		3	5	(25)	(3)	(20)	1	2	20	20	+	+	+	+	+	+	+	‖c
11325	I	3	8	15		20	20	+c	15	5	(30)	(20)	(20)	(30)	(30)	1	1	3			
4668	H	(15)			1	1	2	(2)	(2)	(5)	(20)	2	3	8	8	+c	1				
4565	H			(5)	(8)	(8)	(15)	2 (20)	3	10	4	+H	++	‖c	‖c						

II Relapses

Squirrel number	Spirochæte type	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
4311	H	(20)		(6)	(5)		(10)	(8)				1	5 (10)	(3)		1 (2)					
4531	H	2		(6)				1 (1)	(5)	(3)	(3)	3	3	1	4	3	5				
4905	H	1	1	(25)		(5)	(3)			(2)	(20)	2	(10)	(20)	(20)	1	2	(5)	(5)	(10)	
5070	H	1 (5)		(25)	(20)	(2)			1 (2)	2	1	(10)	(30)	1	(20)	(2)	(1)	(5)	(1)		

The ordinary + sign to signify the presence of spirochætes in the blood has been omitted to a great extent in this table

The presence of spirochætes in the blood is indicated by a horizontal line The numerals *above* the line denote the approximate number of spirochætes noted *per field* those *below* the line, the total number of spirochætes seen in 100 microscopic fields (*see* note to Charts 2 to 9, page 141)

TABLE IX

Showing the highest number of direct sub-passages recorded for each type of spirochæte without evidence of change of type

Source of strains	Number of sub passages for each type of spirochæte								
	A	B	C	D	E	Γ	G	H	I
Madras	582*	549†	194†	289†	317†	23‡	296*	72*	88*
Punjab 1 and 2	305*	288*							
Harro			38‡						

* = sub passage discontinued   † = strain died out   ‡ = strain changed type.

TABLE X

Giving details of change of type occurring without the intervention of a relapse in the course of routine sub-passage showing the number of the sub-passage at which the change occurred in each case

Change of type From	To	Number of sub passages before change occurred in each case						Total number of cases
A	B	274	39					2
C {	B	59	43	10	56	140	83	} 12
	A	40	39	33	147	6	?	
D {	B	115						} 2
	G	57						
E {	A	35						3
	B	29						
	D	42						
Γ	B	6	11					2
G {	A	10	127	324				} 6
	B	96	321	38				
I	A	81						1

Total number of changes to A 10
,,   ,,   ,,   ,,   ,,   B 16
,   ,   ,   ,   ,,   D 1
,,   ,,   ,,   ,,   ,,   G 1

CHART 1

Giving examples of second relapses (third attacks) caused by different types of spirochæte together with their serological reactions

ATTACKS AND RELAPSES			SEROLOGICAL REACTIONS										
	DAYS OF INFECTION		or *Spirochætes* IN VARIOUS ATTACKS										of *sera* at onset of second relapse
Squirrel number	1 2 3 4 5 10 15 20 25 30	Attack number	Test sera										
			A	B	C	D	E	F	G	H	I	
307	A — B — C	1	+	−	−	−	−					A=1/ 320
		2	−	+	−	−	−					
		3	−	−	+	−	−					B=1/5120
261	B — A — D (died)	1	−	+	−	−	−					A=1/5120
		2	+	−	−	−	−					
		3	−	−	−	+	−					B=1/ 320
288	A — B — E	1	+	−	−	−	−					A=1/ 160
		2	−	+	−	−	−					
		3	−	−	−	−	+					B=1/5120
594	C — A+B — F	1	−	−	+	−	−	−	−			A=1/1280
		2										B=1/ 640
		3	−	−	−	−	−	+	−			C=1/ 640
850	A — B — G	1	+	−	−	−	−	−	−			A=1/ 640
		2	−	+	−	−	−	−				
		3	−	−	−	−	−	−	+			B=1/5120
3703	A — B — H (died)	1	+	−	−	−	−	−	−	−		A=1/5120
		2										
		3	−	−	−	−	−	−	−	+		B=1/1280
7056	C — I	1	−	−	+	−	−	−	−	−	−	
		2	−	−	−	−	−	−	−	−	+	
4637	B — E — C+A	1	−	+	−	−	−	−	−			B=1/2560
		2										
		3	+S	−	+	−	−		−			E=1/1280
2498	E — A — B+F (died)	1	−	−	−	−	+	−	−			
		2	+	−	−	−	−	−				
		3	−	+	−	−	−	+				
1733	B+C A F+G	1	−	+	+S	−	−	−	−			A=1/ 640
		2										B=1/5120
		3	−	−	−	−	−	+	+			C=1/ 40
5070	D / H — ?A — C+F+G	1	−	−	−	+	−	−	−			
		2	+	−	−	−	−	−	−			
		3	−	−	+	−	−	+S	+S			
	F+G (died)	1	−	+S	+	−	−	−	−			B=1/ 640
		2	+S	−	−	−	−	−	−			
		3	−	−	−	−	−	+	+			C=1/5120

he ordinary + sign to si… roch… nate …pirocll… …ote to C

CHARTS 2 TO 9

Showing different forms of agglutination curve following infections with
Sp   carteri

The presence of spirochætes in the blood is shown by the + or − signs   The numbers above the horizontal limb of the cross indicate the intensity of the infection   those to the *left* of the vertical limb give the total number of spirochætes seen in 100 *fields* of the dark ground illumination (1/12th objective)   those to the *right* the number of organisms *per field*

A rough estimate of the actual number of spirochætes represented by these figures can be made from the fact that in two actual counts 10 spirochætes *per field* were estimated at 188,936 and 146,653 organisms per c c of blood

The double and triple *plus* signs indicate that the spirochætes in each field were too numerous to count directly (about 1 to 2 millions and 3 to 4 millions per c c of blood respectively)

The small ' c ' above the plus sign means that spontaneous clumping of the spirochætes occurred in the blood on that day

The serological reactions of the infecting spirochæte are noted under the + sign of the day on which they were tested

CHART 2

Single attack—Curve showing reaction to the infecting type of spirochæte only (Form 1)

Squirrel No 5148C

| Day of infection | 1 2 3 4 5 | 10 | 15 | 20 | 25 | 30 | 35 | 40 |

Spirochætes in Blood   4 2₄ ++ ++ +++ +++ - - - - - - - - -

Serum Titre

40,960	A –
20,480	B –
10,240	C' +
5,120	D –
2,560	E –
1,280	G –

0 2 c c blood from Squirrel No 5126 C

C   C   C   C   C

A —— A —— A —— A —— A —— A
B —— B —— B —— B —— B —— B
C
D —— D —— D —— D —— D —— D
E —— E —— E —— E —— E —— E
G —— G —— G —— G —— G —— G

CHART 3

Single attack—Two curves rising *in succession* showing reactions to the infecting and another type of spirochæte (Form 2)

Squirrel No 5646

| Day of infection | 1 2 3 4 5 | 10 | 15 | 20 | 25 | 30 | 35 | 40 |

Spirochætes in Blood   2₄ ++ ++ +++ +++ ++ - - - - - - -

Serum Titre

A ⌐
B –
C –
D –
E +
G –

| 40,960 |
| 20,480 |
| 10,240 |
| 5,120 |
| 2,560 |
| 1,280 |
| 640 |
| 320 |
| 160 |
| 80 |
| 40 |
| 20 |
| 10 |

0 1 c c blood from Squirrel No 5626 E

E

E

E

A   A   A

A —— A
B —— B —— B —— B —— B
C —— C —— C —— C —— C
D —— D —— D —— D —— D
E
G —— G —— G —— G —— G

CHART 4

Single attack—Two curves rising *simultaneously* showing reactions to the infecting and another type of spirochæte (Form 3)

Squirrel No 5323B

| Day of infection | 1 2 3 4 5 | 10 | 15 | 20 | 25 | 30 | 35 | 40 |

Spirochætes in Blood

Serum Titre

A –
B +
C –
40,960   D –
20 480   E –
10 240   G –
5 120
2 560
1,280
640
320
160
80
40
20
10

0 2 c c blood from Squirrel No 5307 B

CHART 4a
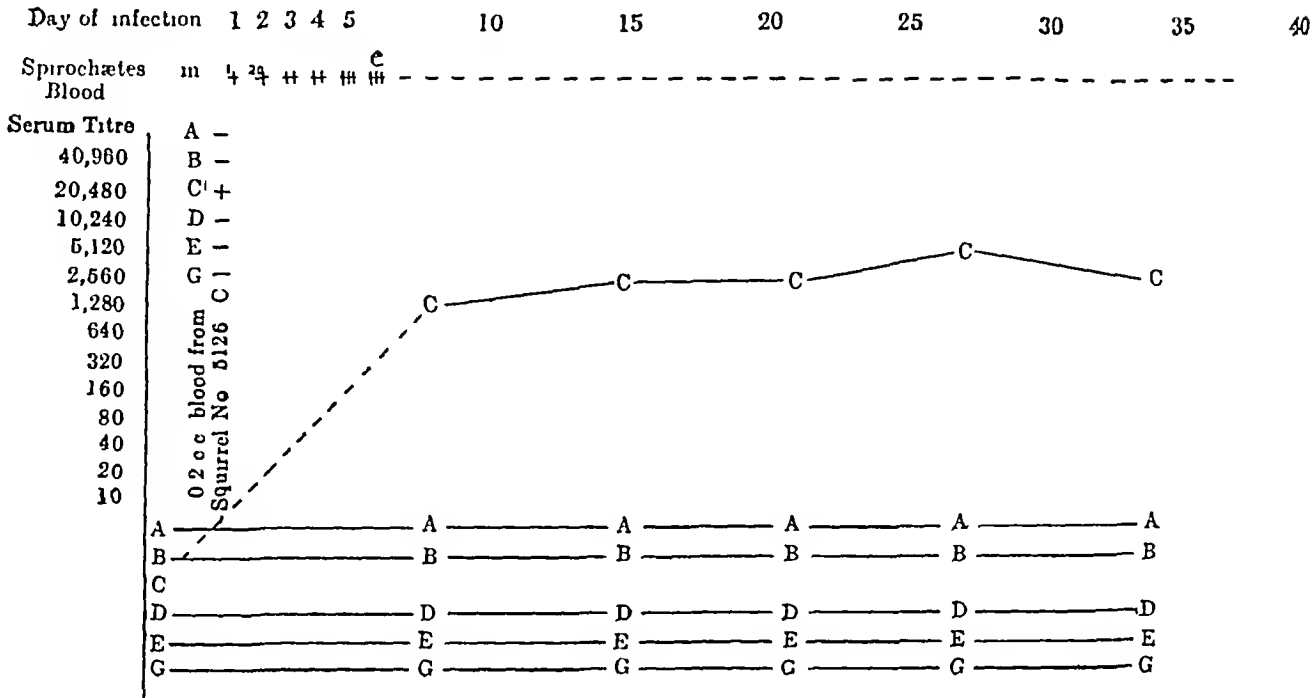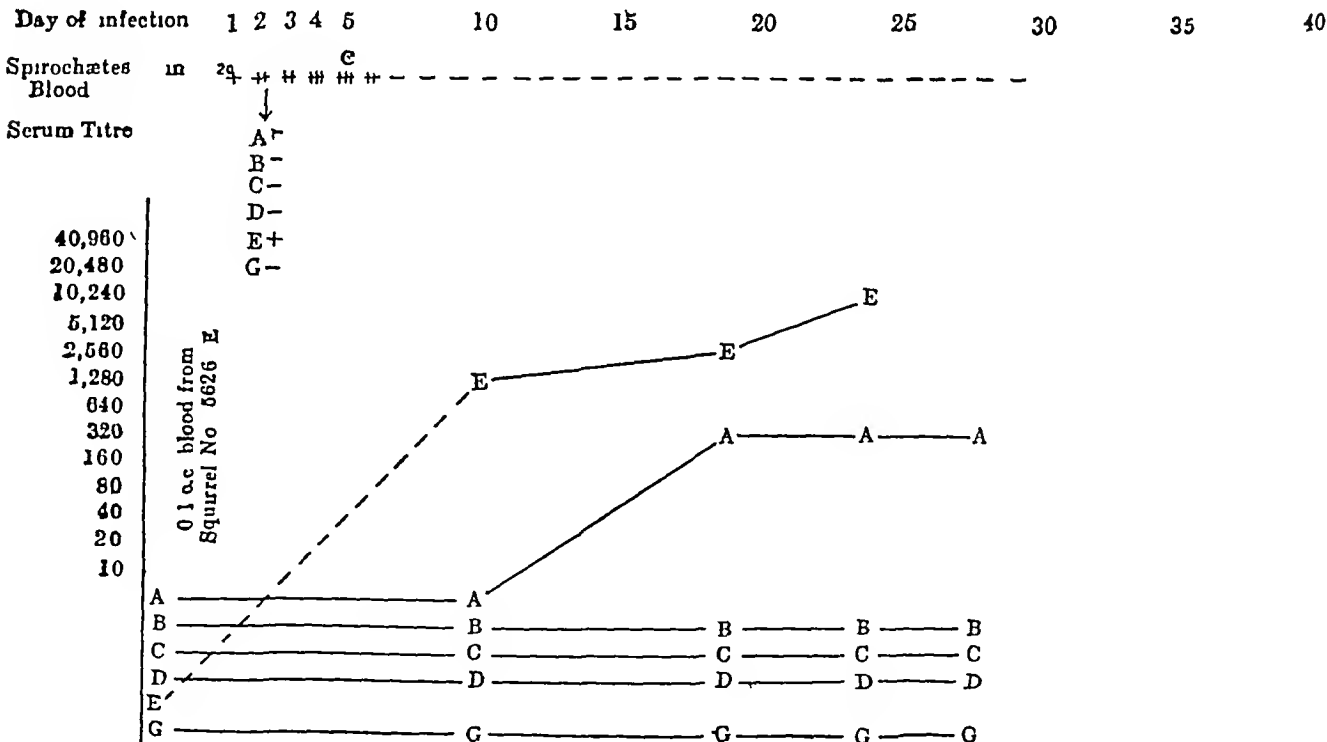
Single attack—Five curves rising *simultaneously* showing reactions to the infecting and four other types of spirochæte (Form 3)

Squirrel No 5728D

| Day of infection | 1 2 3 4 5 | 10 | 15 | 20 | 25 | 30 | 35 |

Spirochætes in Blood

Serum Titre

A –
B –
40 960   C –
20 480   D +
10 240   E –
5 120   G –
2 560
1 280
640
320
160
80
40
20
10

0 2 c c blood

CHART 2

Single attack—Curve showing reaction to the infecting type of spirochæte only (Form 1)

Squirrel No 5148C

Day of infection	1	2	3	4	5	10	15	20	25	30	35	40

Spirochætes in Blood   ᵻ ²ᵻ ++ ++ +++ C/+++ – – – – – – – – – – – –

Serum Titre												
40,960	A –											
20,480	B –											
10,240	Cʰ +											
5,120	D –											
2,560	E –											
1,280	G –											
640												

0 2 c c blood from Squirrel No 5126 C

A
B
C
D
E
G

CHART 3

Single attack—Two curves rising *in succession* showing reactions to the infecting and another type of spirochæte (Form 2)

Squirrel No 5646

Day of infection	1	2	3	4	5	10	15	20	25	30	35	40

Spirochætes in Blood   ²ᵻ ++ ++ +++ C/+++ ++ – – – – – – – – – –

Serum Titre

A ⊦
B –
C –
D –
E +
G –

40,960										
20,480										
10,240										
5,120										
2,560										
1,280										
640										
320										
160										
80										
40										
20										
10										

0 1 c c blood from Squirrel No 5626 E

A
B
C
D
E
G
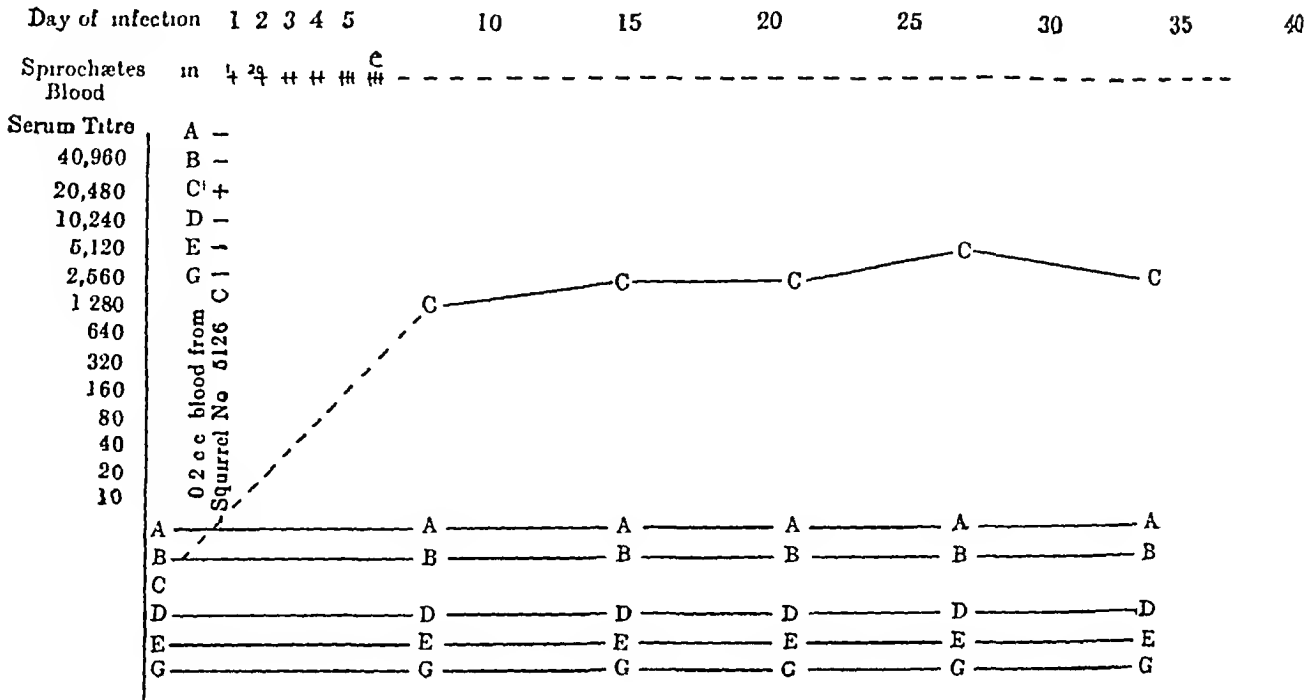
Chart 6

First attack and one relapse—Curves showing reactions to infecting and 'relapse' spirochætes only (Form 1 in relapse)

Squirrel No 5838D

Day of infection 1 2 3 4 5   10   15   20   25   30   35

Spirochætes in Blood   + + ++ ++ +++ +++ +++ — — — + + +++ +++ +++ — — — — — —

Serum Titre
A —   A +
B —   B —
C —   C —
D +   D —
E —   E —
G —   G —

40 960
20 480
10 240
5 120
2 560
1,280
640
320
160
80
40
20
10

0 3 c c blood from Squirrel No 5808 D

Chart 7

First attack and one relapse—Curves showing reactions to infecting and 'relapse' spirochætes the latter followed by a further reaction to another type (Form 2 in relapse)

Squirrel No 5505B

Day of infection 1 2 3 4 5   10   15   20   25   30   35   40

Spirochætes in Blood

Serum Titre
A —
B +
C —
D —
E —
G —

40 960
20 480
10,240
5 120
2 560
1,280
640
320
160
80
40
20
10

0 2 c c blood from Squirrel No 5485 B

J, MR

10

CHART 8

First attack and one relapse—Curves showing reactions to infecting and 'relapse' spirochætes reactions to two other types occurring *simultaneously* with that of the 'relapse' spirochæte (Form 3 in relapse)

Squirrel No 4995B.
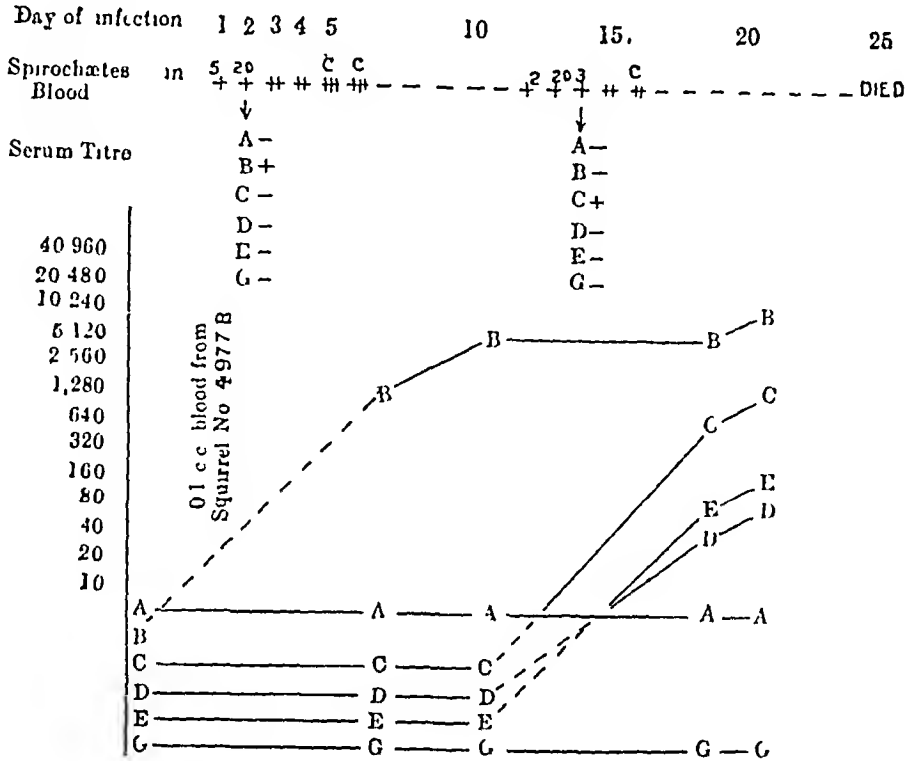
Day of infection 1 2 3 4 5   10   15,   20   25

Spirochætes in 5 20   C C   2 20 3   C   DIED
Blood   + + ++ ++ +++ ++ — — — — — + + + ++ ++ — — — — — —

Serum Titre

A —	A —
B +	B —
C —	C +
D —	D —
E —	E —
G —	G —

40 960
20 480
10 240
5 120
2 560
1,280
640
320
160
80
40
20
10

01 cc blood from Squirrel No 4977 B

CHART 9

First attack and one relapse—Curves showing reactions to infecting and 'relapse' spirochætes reactions to two other types (1) following first attack, (2) occurring *simultaneously* with relapse (Form 2 in first attack and Form 3 in relapse)

Squirrel No 5880D

Day of infection 1 2 3 4 5   10   15   20   25   30   35

Spirochætes in 15 30   C C   3 3 10   C
Blood   + ++ ++ ++ ++ ++ — — — — — + + + ++ ++ — — — — — —

Serum Titre

A —	A +
B —	B —
C —	C —
D +	D —
E —	E —
G —	G —

40,960
20,480
10 240
5,120
2,560
1,280
640
320
160
80
40
20
10

01 cc blood from Squirrel No 5873 D
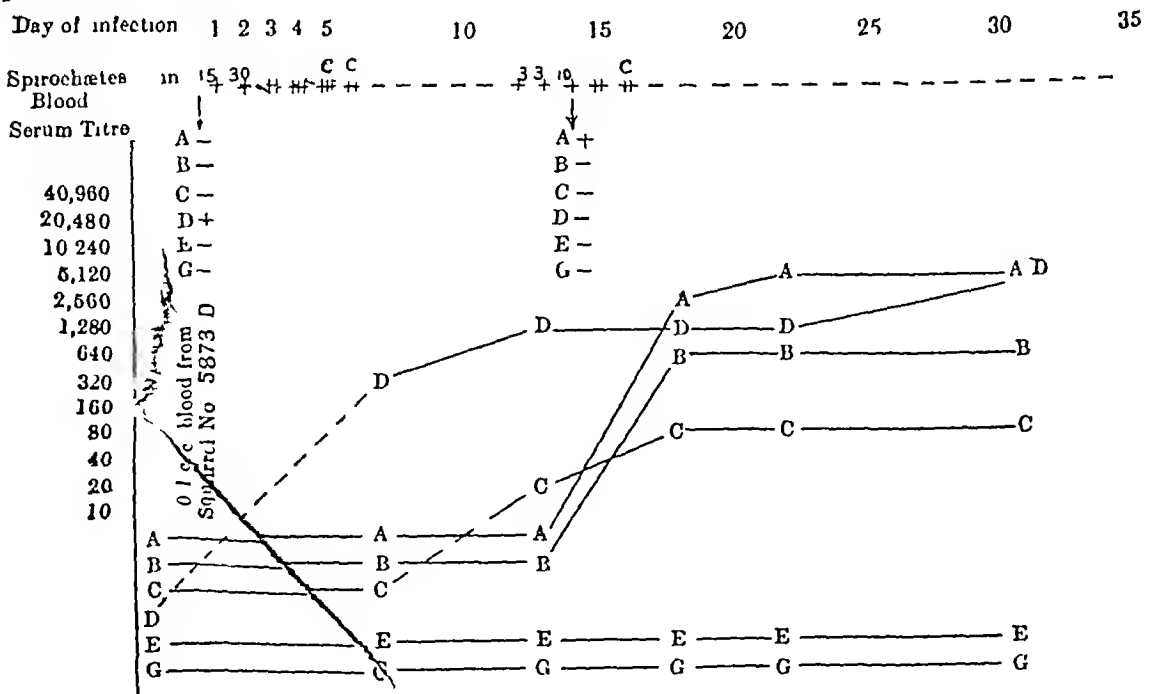
CHARTS 10 TO 18

*Showing the types of spirochæte found in relapses arising from ' P_1,' ' P_2 '
and ' Hazro ' strains and also from different types isolated
from second relapses*

In the following charts the types of spirochæte found in relapses arising from the parent stem or in a succession of relapses are shown in diagrammatic form

The *vertical* lines represent sub passages in the direct line carried out during the first attack   the *horizontal* lines, the relapses arising from these

The *letters* refer to the types of spirochæte found

The *numerals* refer to the number of generations the organism has passed through since its isolation from the human being

Sub passages in which the type of spirochæte responsible for attacks or relapses has not been examined have been omitted

CHART 8

First attack and one relapse—Curves showing rea〈
spirochætes reactions to two other type〈
with that of the 'relapse' spirochæte〈

Squirrel No 4995B
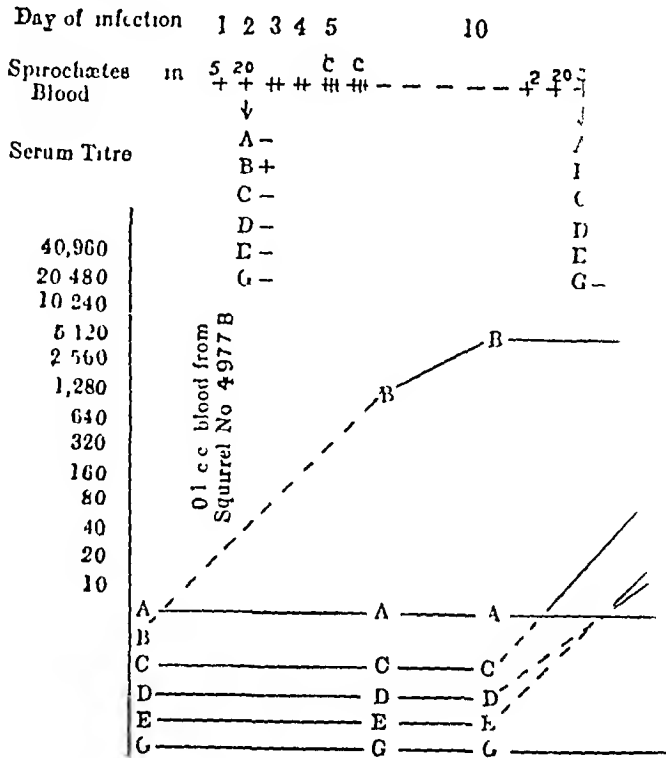
CHART 9

First attack and one relapse—Curves showing reactions t〈
spirochætes reactions to two other types (1) follow
occurring *simultaneously* with relapse (Form 2
and Form 3 in relapse)

Squirrel No 5880D
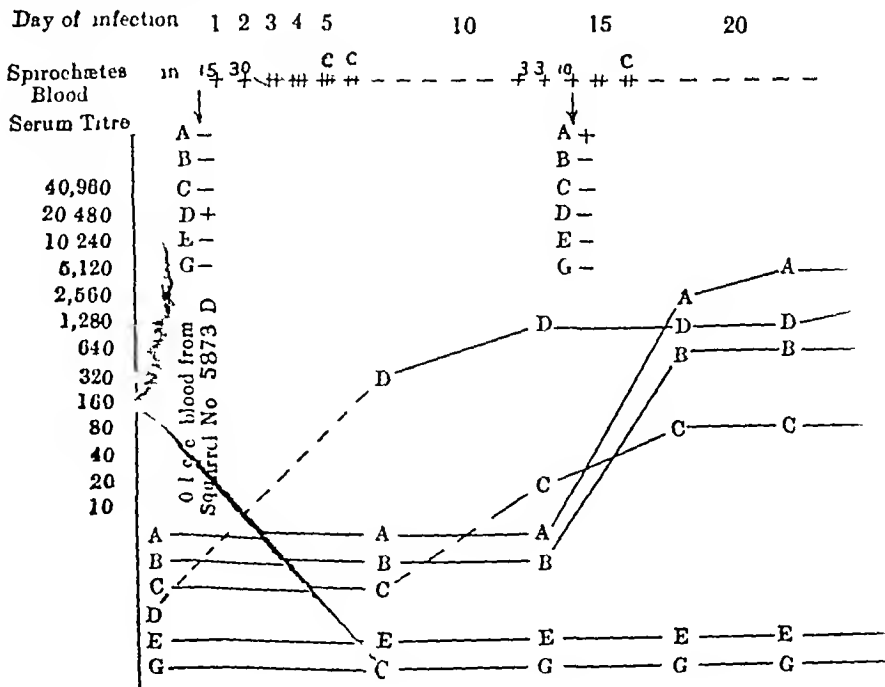
CHART 12

Showing the types of spirochæte found in relapses occurring in the Punjab strain 'Hazio' ('C' type)

PASS AGE Nº	SERIES I	II	III	IV
	HUMAN C			
1	4 ———— A			
	5 ———— B	15 ———— B		
10	6 ———— A	16 ———— C		
	13 ———— A	10 ———— B		
	15 ———— BA	13 ———— B		
20	20 ———— B	18 ———— B		
		23 ———— B		
		24 ———— B		
		25 ———— B		
		26 ———— B		
		27 ———— B		
30	28 ———— D	28 ———— B		
	31 ———— B	31 ———— B		
		32 ———— B		
		34 ———— B	34 ———— AG	
40	CHANGED TO A	37 ———— B		
	39 ———— B			
	40 ———— B			
	41 ———— B			
	45 ———— B	42 ———— C		
	46 ———— B			
50		52 ———— B		
		53 ———— B		
		56 ———— B		
		57 ———— B		
	58 ———— B	58 ———— B		
	59 ———— D	59 ———— B		
60		60 ———— D		
		61 ———— B		
	66 ———— B			
	67 ———— BC			
70	69 ———— B			
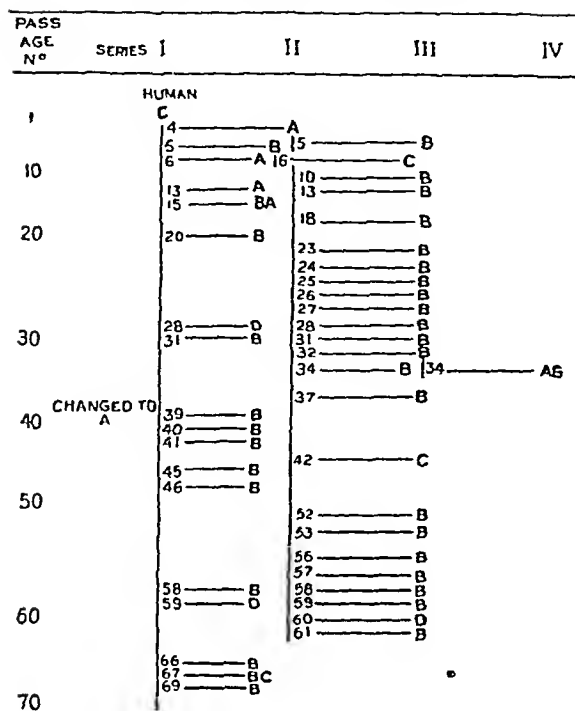
CHART 13

Showing the types of spirochæte found in a succession of relapses originating in type 'C'

SERIES	I	II	III	IV
	396B — A — C			
	398 ———— B			
	400 ———— AB			
		401 ———— A	403 ———— B	
		404 ———— A		
		405 ———— A	405 ———— B	
	405 ———— A			
	406C 406A			
	407 ———— A	407 ———— B		
		409 ———— B		
		417A		
	409 ———— A			
	410C	411 ———— C		
	412C			
	413 ———— A			
	417 ———— A			
	422C			
	423C			
	431C			
	432C			
	440C			
	441C			
	442CGs			
	447 ———— A			

CHART 14

Showing the types of spirochæte found in a succession of relapses originating in type 'D'

SERIES	I	II	III

```
392B ——— A ——— D
                  |393 ——————— B
                  |            |395 ——————— A
                  |394 ——————— B    397A
                  |            |395 ——————— A
                  |            |396 ——————— A
                  |            398B
                  |398 ——————— B
                401D
                405DBs
                406D (NO B ELEMENTS)
                422DB ——————— A
                  |            |423 ——————— B
                  |424 ——————— A
                  |426 ——————— A
                  |428 ——————— A
                431D ——————— A
                  |432 ——————— ABs
                445D
                450G
                458G
                460G
```

CHART 15

Showing the types of spirochæte found in a succession of relapses originating in type 'E'

SERIES	I	II	III	IV

```
391A   D   E
               |394 ——————— A
               |            |404 ——————— E
               |            |            |409 ——————— A
               |            |            |411 ——————— A
               |            |            |412 ——————— Q
               |            |            416FBs       420C
               |            |            |417 ——————— A
               |            |            |418 ——————— A
               |            |            419F
               |            |            B
               |            |405 ——————— B
               |            |            |408 ——————— A
               |            |414 ——————— B
               |            |416 ——————— B
               |            |            |417 ——————— G
               |            |417 ——————— B
               |            |421 ——————— B
               |411 ——————— A
               |            |417 ——————— E
               |            |419 ——————— E
               |415 ——————— ABs
               |416 ——————— B
               |            |416 ——————— A
               |            |420 ——————— A
               |            |423 ——————— A
               |            |425 ——————— D
               |            |425 ——————— D
               |            |429 ——————— D
               |417 ——————— DAsBs
               421E
               |423 ——————— D
               432E
               457E
               458EBs
               |493 ——————— B
               |            |494 ——————— A
               |            |            |495 ——————— C
               |            |            |            |500 ——————— A
               |            |            |            |501 ——————— A'
               |499 ——————— A            503C
               504E                      |506 ——————— A
               510E
               |519 ——————— A
               523E
               |530 ——————— A          539CASBs
               531E                     547C
               566E                     554C
               573E                      |561 ——————— AGs
               579E                      |563 ——————— A
       |582 —|580 ——————— D             |564 ——————— A
       |      |584 ——————— A           567C
       591E   |590 ——————— A            |585 ——————— A
              |592 ——————— ABs          |588 ——————— AGs
              |            ABs         588C
              |602 ——————— G            |593 ——————— A
       |606 —|            |609 ——————— DDs  |594 ——————— A
       |609 —|— D                        |600 ——————— AGs
       |610 —|— D                        |607 ——————— A
       |611 —|— D                        |608 ——————— A
       |613 —|— D                      616C
       617E                             |619 ——————— A
       |618 —|— D
       |622 —|— D
       622E                            620C
       |624 —|— D                       |624 ——————— A
       639E
       |645 ——————— B                  CHANGED TO
       645E |647 ——————— A              a B
       |650 ——————— A
       654E
       706E
STRAIN DIED
   OUT
```

CHART 16

*Showing the types of spirochæte found in a
succession of relapses originating
in type 'F'*

SERIES	I	II	III	IV

```
407C —— AB ——FDs
        409FDsEs
            |410 ———————— B
            |             1412 ———————— C
            |411 ———————— B
            |             {412 ———————— A
            |             |414 ———————— A
            |             {417 ———————— A
            |             423BD
            412FDs ———————— B
            |413 ———————— B
            |             1414 ———————— A
            414F
            |417 ———————— C
            |             419C ———————— BC
            |418 ———————— E                    |
            |             421 ———————— A    |424 ———————— A
                                          429B
                                          430B
```
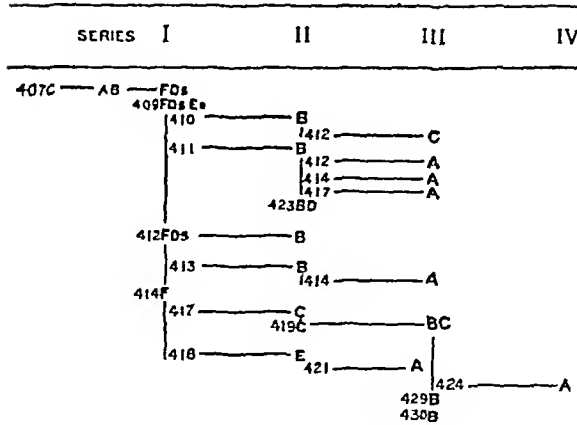
CHART 17

*Showing the types of spirochæte found in a succession
of relapses originating in type 'G'*

SERIES	I	II	III	IV	V

```
417A-- B---G
        |420 ———————— CBs
        |421 ———————— BCs
        424G ———————— F
        425G
        426G                  |428 ——————— A
        429G                  |429 ——————— D
                    430B      430D
                              432D
        434 ——— E  |          435D
                434B
                     |437 ——— D  |
        439 ——— C              438D ———————— G
                441B
                              |447 ———————— B
        450GFs                450D
                              |458 ———————— A
                              |460 ———————— A
                              461D ———————— A
                              476D ———————— B
                              |477 ———————— B
                              |478 ———————— A
                              |480 ———————— A
                              |481 ———————— BF
                              |482 ———————— A
                              483D
                              488D
                              494DA2Bs
                              |497 ———————— A
                              |499 ———————— A
                              500D ———————— A
                              |501 ———————— A
                              502D           A
                              |505 ———————— A
                              507D
                              |508 ———————— G
                                           |510 ———————— BFAB
                              CHANGED       |511 ———————— BF
                              TO B          |512 ———————— BF
                                           513G
                              
                              CHANGED
                              TO A₁
```

Chart 19

Distribution curves showing lengths of first attacks in (1) types 'A' and 'B' and (2) type 'H'

DAYS	TYPES 'A' AND 'B' ACTUALS	%	TYPE 'H' ACTUALS	%
1	1	0.2	1	0.7
2			2	1.3
3	1	0.2	2	1.3
4	3	0.5	2	1.3
5	44	7.1	7	4.7
6	459	74.6	37	25.0
7	88	14.3	11	7.4
8			10	7.0
9	14	2.3	4	2.7
10	4	0.6	4	2.7
11	1	0.2	12	8.1
12			8	5.4
13			14	9.4
14			7	4.7
15			12	8.1
16			3	2.0
17			8	5.4
18			3	2.0
19			3	2.0
TOTAL	615		148	

PER CENTAGE: 70, 60, 50, 40, 30, 20, 10

DAYS: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

TYPE 'H' ·—·—·   TYPES 'A' & B +—·—·

Chart 18

Showing the types of spirochæte found in a succession of relapses in a series of second relapse strains

SQUIRREL Nº	SERIES	I	II	III	IV
1036		416B—A—C 418C 428C 430C		FG |432 —?— |433——A	
983		418F—?—E |421	A		
1733		447BC s—A—FG 448FG |449	B		
1771		485B—A—E |487	A		
1824		448FG—?—B |449	A		
2475		556A—B—C 538C 556 558 559	A A A		
2498		519E—A—B 520B |526	A		
2533		502D—A—CF5Gs |506	AB		
3497		579E—?—A |581 |582 |583	B B B		
3703		222A—B—H |232 |233	EGs EGs		
4637		663B—E—C |664 670 673 674 679	A B B B B		

CHART 20

*Showing a change of type from type
'C' to type 'B' without the
intervention of a relapse*

PASSAGE
Nº

466   C

470   C

475   C   C
          C   C
        BC   B
        B   B
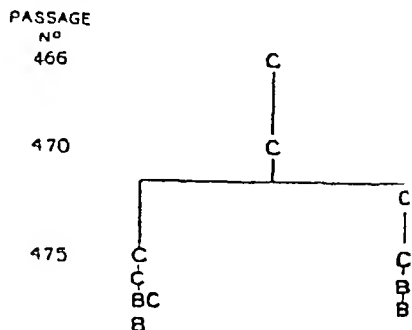
CHART 21

*Showing a succession of changes to other types from
type 'C' in the course of sub-passage
carried on during the first attack*

PASSAGE
Nº

620   C

625   B $C\frac{3}{4}B\frac{1}{4}$ ← B SERUM
                C ← B SERUM

     (27 PASSAGES HERE)

655   C
     E SERUM → $C\frac{3}{4}E\frac{1}{4}$
          C   C
    E SERUM →   A

660   C

665   E }
      A } SERUM → $C\frac{1}{2}A\frac{3}{4}E_{7}$
            $C\frac{3}{4}A\frac{1}{4}$
            C
   E }
     A } SERUM →
   E }
     A } SERUM → $C\frac{3}{4}A\frac{1}{4}$

670   E } SERUM →
     A }
   E } SERUM → C
     A }   C

675   C
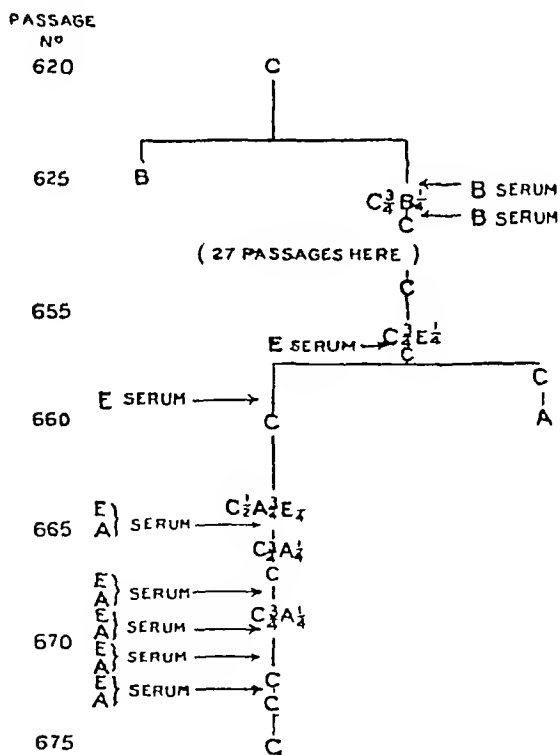
(Strain died out in 12 passages)

CHART 22

Showing change of type from type 'D' to type 'B' occurring during the course of sub-passage during the first attack

PASSAGE
NO

532

D — D — D

535

D — D — D

540 B SERUM → $B\frac{3}{4}D\frac{1}{4}$ $B\frac{1}{2}D\frac{1}{2}$ ← B SERUM

$B\frac{1}{4}D\frac{3}{4}$ ←B SERUM $B\frac{3}{4}D\frac{1}{4}$

D — $B\frac{1}{2}D\frac{1}{2}$ ←B SERUM $B\frac{3}{4}D\frac{1}{4}$ D

D — $D\frac{3}{4}B\frac{1}{4}$ ← B SERUM D

545   D B D D

    D B D
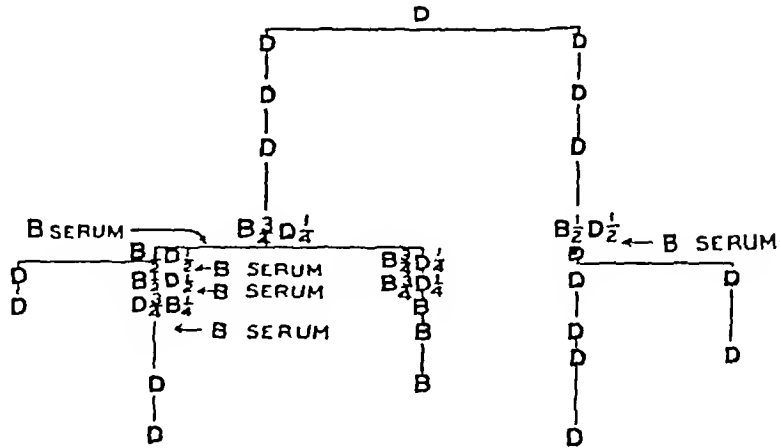
CHART 23

Showing a succession of changes from type 'E' to other types occurring during the course of sub-passage during the first attack

PASSAGE
NO

809
810    $E\frac{3}{4}D\frac{1}{4}$

D SERUM → $E\frac{1}{2}D\frac{1}{2}$ $E\frac{3}{4}D\frac{1}{4}$

E — D SERUM → E — $E\frac{3}{4}D\frac{1}{4}$

815   E E E E

               $E\frac{3}{4}D\frac{1}{4}$

E $E\frac{3}{4}D\frac{1}{4}$

820   E E

(5 PASSAGES HERE)

825   E $E\frac{1}{2}D\frac{1}{2}$

D $D\frac{3}{4}E\frac{1}{4}$

      E

(7 PASSAGES HERE)

835   E E

840   B B SERUM → $E\frac{3}{4}B\frac{1}{4}$

B SERUM → $E\frac{3}{4}B\frac{1}{4}$ B

$E\frac{3}{4}A\frac{1}{4}$ E

845   A E

CHART 24

*Showing a succession of changes from type 'G' to other types
occurring during the course of sub-passage
during the first attack*

PASSAGE
N°
599
600

G

G
G
G
$G\frac{1}{2}B\frac{1}{4}$
B

$G\frac{1}{4}B\frac{3}{4}$
G
$G\frac{1}{2}B\frac{1}{2}$
G

$G\frac{1}{2}B\frac{1}{2}$ ← B SERUM
$G\frac{1}{2}B\frac{1}{2}$ ← B SERUM

605

B SERUM →
B SERUM →
B SERUM →

G
G

$G\frac{1}{2}B\frac{1}{2}$ ← B SERUM
$G\frac{1}{4}B\frac{3}{4}$
$G\frac{1}{4}B\frac{3}{4}$

G
$G\frac{3}{4}A\frac{1}{4}$ $G\frac{3}{4}A\frac{1}{4}$ G ← B SERUM

610

G

CHART 25

*Showing a succession of changes from type 'G' to other types
occurring during the course of sub-passage
during the first attack*

PASSAGE
N°
608

G
$G\frac{3}{4}A\frac{1}{4}$
A SERUM →

610

$G\frac{3}{4}A\frac{1}{4}$
G
G G   A SERUM → $G\frac{3}{4}A\frac{1}{4}$ G
G

$G\frac{3}{4}A\frac{1}{4}$
$G\frac{3}{4}A\frac{1}{4}$

G
G
G G   G

615

G

$G\frac{3}{4}A\frac{1}{4}$
$G\frac{3}{4}A\frac{1}{4}$

G
G
G
G

620

G G

$G\frac{1}{2}A\frac{1}{2}$
$G\frac{1}{2}A\frac{1}{2}$

G

625

630

$A\frac{3}{4}G\frac{1}{4}$

635

A

(4) Acetylation did not precipitate or inactivate oxytocin

(5) The oxytocic hormone could not be extracted with ether in an alkaline medium

(6) The oxytocic hormone was inactivated by hydrolysis with H_2SO_4 for ten minutes

(7) The sodium phosphotungstate fraction of the pituitary extract was practically free from histamine as it was almost entirely inactivated by treatment with 2NNaOH for one hour at room temperature

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THE INFLUENCE OF ADRENALINE, PITUITARY EXTRACTS AND INSULIN ON THE MOVEMENTS OF THE INTESTINE

BY

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The influence of adrenaline, pituitary extracts and insulin on intestinal movements was studied in about 30 cats   The drugs were either injected intravenously into chloralosed animals or added to the bath of oxygenated Locke's fluid at 38°C in which excised segments were suspended   The movements of the intact bowel were recorded by the balloon method and those of excised segments by Brodie's levers attached by thread to the upper ends of the segments suspended in the bath

Adrenaline —Adrenaline is said to cause generally, on injection into the body or when it is secreted in large amount in the body, inhibition of the intestinal movements and resembles sympathetic stimulation in its effects   But there have been sporadic reports that adrenaline may have a motor effect on the intestine   Salvioli (1902), Magnus (1903), Hoskins (1912), Sharpey-Schafer (1924, 1926), all reported excitatory effects   Takeda (1930) showed that, after pilocarpine or physostigmine, adrenaline caused no relaxation   Bernheim and Blockson (1932) observed that, after pilocarpine or physostigmine, adrenaline caused greater contraction in isolated strips followed after some time by slow relaxation   Templeton and Lawson (1932) stated that data were accumulating to show that adrenaline stimulates not only the thoracico-lumbar but also the cranio-sacral apparatus

In my experiments, adrenaline, 0 1 c c or 0 2 c c of 1 in 1,000 solution given intravenously, or $\frac{1}{4}$ c c to 1 c c of 1 in 1,000 solution added to the bath of 100 c c containing the excised segment, usually caused inhibition of the movements   A previous dose of ergotoxine up to 10 mg had no influence on the inhibitory effect of adrenaline on the intact bowel, although it caused a reversal effect of adrenaline on blood-pressure   Following a dose of pilocarpine (1 mg to 5 mg), adrenaline caused only slowing of the contractions both in the intact bowel and in excised segments suspended in a bath   In one animal, after vagal stimulation, adrenaline given intravenously caused a marked increase in tonus and prolonged powerful contraction of the bowel as shown in Fig 1 of the Graph   Following histamine or barium chloride, adrenaline caused only the usual inhibitory effect

These results show that, whenever there is increased parasympathetic tone following either vagal stimulation or administration of pilocarpine, adrenaline

causes either increased tonus and contraction or has only a slowing effect The findings of Takeda, and Bernheim and Blockson are borne out by these results

Stimulation of the splanchnics under similar conditions i c , following vagal stimulation or injection of pilocarpine, had also a slowing and not a complete inhibitory effect as shown in Figs 2 and 3 of the Graph Kuré Ichiko and Ishikawa (1931) reported that the greater splanchnic and the lumbar sympathetic trunk in dog or cat contain, besides, the well-known inhibitory fibres many excitatory fibres Barry (1932) also found that stimulation of one of the split branches of the great splanchnic caused increased activity with or without preliminary inhibition The absence of inhibition on stimulation of the splanchnic after vagal stimulation or injection of pilocarpine into the body may, therefore, be interpreted as due to the presence in the splanchnic of a varying number of excitatory fibres whose activity, under conditions of increased parasympathetic tone, effectively counteracts the action of the inhibitory fibres

Just as in the case of blood-vessels, adrenaline brings about vaso-dilatation when the vaso-constrictors are put out of action by ergotoxine, just as in the pregnant condition of the uterus in some animals, adrenaline causes contraction instead of inhibition owing to the activity of the excitatory fibres of the sympathetic supplying that organ, so also in the case of the intestine it is highly probable that adrenaline has no inhibitory effect or may even cause contraction owing to the activity of the excitatory fibres under conditions of increased parasympathetic tone It need not, therefore, be assumed, as done by Templeton and Lawson (1932), that adrenaline may stimulate both the cranio-sacral and the thoracico-lumbar outflows

Pituitary extracts —As early as 1909, Blair Bell and Hick noted that extracts of the pituitary posterior lobe stimulated strips of muscle from the small intestine Since then it has been generally held that pituitrin has a stimulating effect on the intestinal muscle and could be used to improve the tone of the bowel in cases of atony But reports that pituitrin is capable of producing inhibition of the intestinal movements have been made by Hoskins (1916), Shamoff (1916) and Degener (1922) Gruber and Robinson (1929) found in unanæsthetized animals that pituitrin caused series of waves of contractions intermittent with loss of tone Carlson (1930) found pituitrin had a stimulating effect on the human intestine but the effects were inconstant in dogs, a stimulating effect being the exception Elmer *et al* (1930) found in the rabbit that oxytocin depressed the intestine while vaso-pressin augmented the movements Quigley and Barnes (1930) found that all pituitary preparations invariably produced inhibition of the gastro-intestinal tract of dogs without any indication of augmentation

In my experiments on cats, pituitrin given intravenously in doses varying from 1 to 10 units caused a primary transient augmentation followed by complete inhibition of the movements of the bowel When pituitrin was added to the bath containing the isolated bowel, there was definite inhibition sometimes preceded by a slight transient augmentation The active principle oxytocin (Pitocin of P D & Co) 1 to 5 units given intravenously caused an augmentation lasting for more than a minute and then inhibition, while vaso-pressin (Pitressin of P D & Co) caused a primary augmentation similar to that caused by pituitrin followed by gradual inhibition The effects of the active principles on the isolated bowel were also similar

Fig 1
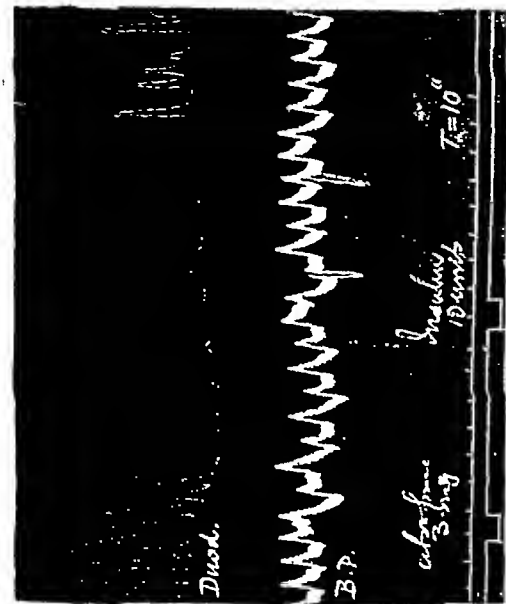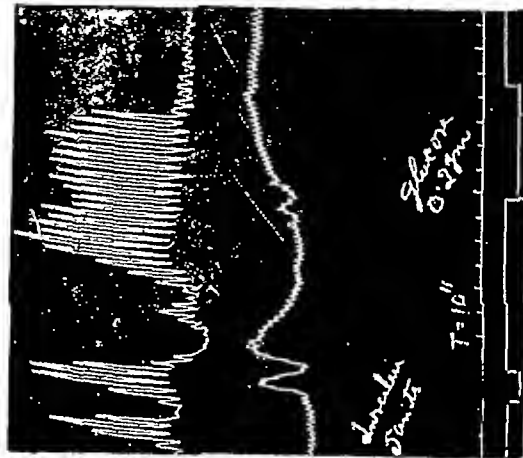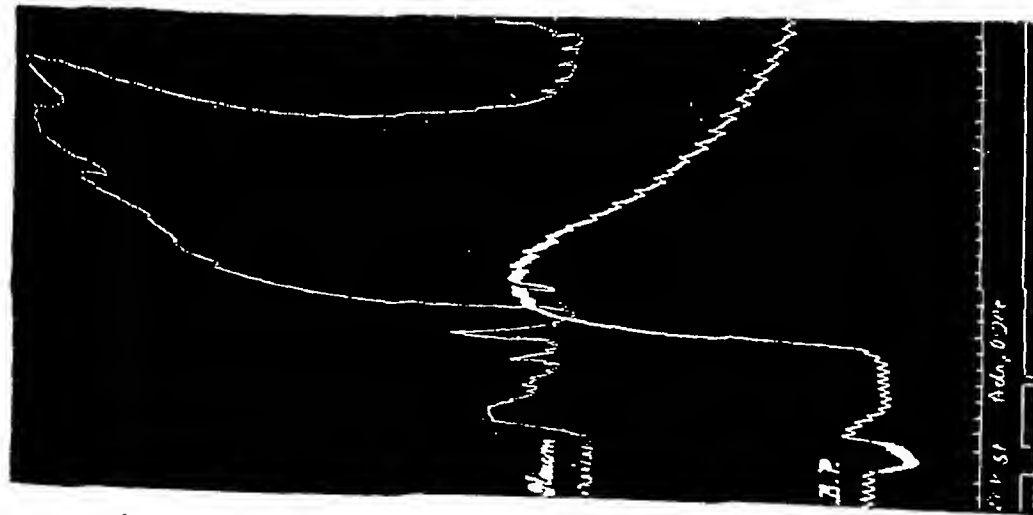
Fig 1 shows the effect of adrenaline (0 2 c c of 1 in 1,000 solution) given intravenously, on the movements of the ileum of a chloralosed cat, following vagal stimulation   Fig   2 shows the absence of inhibition of the movements of the duodenum, recorded by the balloon method, when the left splanchnic was stimulated with a weak current after vagal stimulation in a chloralosed cat   Fig   3 shows the absence of inhibition of the movements of the duodenum and ileum, recorded by the balloon method, when, in a chloralosed cat, the left splanchnic was stimulated with a strong current after pilocarpine 1 mg given intravenously   Fig   4 shows the effect of intravenous injection of atropine (3 mg) on the insulin contractions of the duodenum of a chloralosed cat and the effect of a further dose of insulin (10 units) given intravenously during the inhibition of the movements brought about by atropine   Fig 5 shows the

It is concluded from these observations that pituitrin and its active principles have an initial augmentative effect on the bowel of cat followed by sudden or gradual inhibition   The primary augmentation is more prolonged with oxytocin   The effects of pituitary extracts on the bowel seem to vary in different animals but, in general, it may be said that the extracts cannot be effective intestinal stimulants, in as much as inhibition sets in suddenly or gradually after the augmentation if there is any

Insulin —The results of insulin administration, in my experiments on chloralosed cats, corroborated the findings of Quigley and Solomon (1929) in man and those of Quigley and Barnes (1930) in dogs   Insulin in doses of 1 to 5 units given to starved animals or 5 to 25 units given to animals that were not starved had a stimulating effect on the movements of the bowel   When the vagi were severed during the period of insulin excitation, there was sudden inhibition due to the loss of vagal tone but the movements soon reappeared and a further dose of insulin had again a stimulating effect   Atropine 3 mg given intravenously caused complete inhibition of the insulin movements but with a further dose of insulin (10 units) contractions reappeared after a delay of one minute as in Fig 4 of the Graph   Glucose given intravenously in one dose of 0 2 g or sometimes in 2 or 3 doses of 0 2 g each inhibited the insulin movements as in Fig 5 of the Graph and a further dose of insulin had no effect

These experimental data show that insulin acts on the intestinal muscle indirectly by lowering the sugar content of the blood and not by direct action on the vagal centre or on the vagal endings   Insulin had no effect on the isolated bowel suspended in a bath of oxygenated Locke's fluid at 38°C

Summary

1   The findings of Takeda (1930) and of Bernheim and Blockson (1932) regarding the excitatory effects of adrenaline under conditions of increased parasympathetic tone are confirmed   The excitatory effects under such conditions are considered to be due to the influence of the excitatory fibres of the sympathetic found by Kuré, Ichiko and Ishikawa (1931) and by Barry (1933)

2   It is found that pituitrin and its active principles have an initial augmentative effect followed by sudden or gradual inhibition, on the bowel of the cat, and experimental evidence is quoted to show that the effects vary in different animals

3   The effects of insulin on the bowel found by Quigley and Solomon (1929) in man and Quigley and Barnes (1930) in dogs are confirmed in cats   It is found that insulin stimulates the bowel indirectly by lowering the sugar content of blood

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PREPARATION, PROPERTIES AND PHARMACOLOGICAL ACTION OF ANHYDROCOTARNINE-CARBAMIDE

BY

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RECENTLY several attempts have been made to utilize the narcotine, which is obtained in considerable quantities as a by-product in the manufacture of morphine from Indian opium, by the preparation of such derivatives as might have anti-malarial and anti-pyretic properties. The pharmacological action and toxicity of one of these derivatives, anhydrocotarnine-resorcinol (Liebermann and Glawe, 1904) originally prepared by Liebermann, have been carefully studied by Grewal, Kochhar and Ray (1933) under the name of 2′ 4′-dihydroxyphenyl-hydrocotarnine hydrochloride, and also by Chopra, Mukherji and Campbell (1933) under the original name given by Liebermann, but both these groups of workers have found the drug to be devoid of any prophylactic or curative action whatever either in malarial or other fevers.

The well-known physiological activities of carbamide derivatives led us to investigate the reaction between urea and narcotine as well as cotarnine, the resulting compounds being expected to develop important medicinal properties. Though our expectations have not been fully realized, certain interesting results have been obtained which are described in the present communication.

The action of urea on narcotine at high temperatures has been studied by von G. Frerichs (1903). Repetition of his work under several different conditions led to

the isolation of [a colourless crystalline substance melting at a temperature of 102°C , the yield of the product was, however, very poor, rarely exceeding 10 per cent of the weight of the narcotine employed   It is proposed to undertake a detailed examination of the pharmacological action of this compound as soon as its structure has been determined with certainty and the yield of the product materially improved

The reaction between urea and cotarnine takes place smoothly and quantitatively, the product crystallizing in colourless rectangular prisms melting at 182°C   From its analyses and general properties, the substance, which has been named '*Anhydrocotarnine-carbamide*', may be assigned either of the two following structures ·—

'A'   'B'
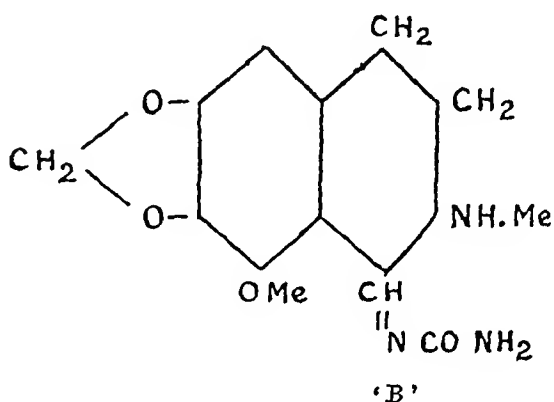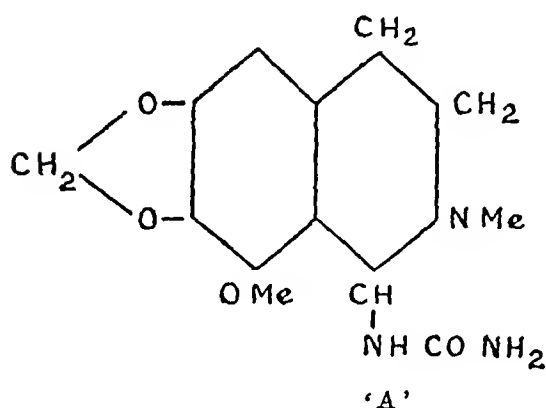
The formula ' A ' brings it into line with the constitutions proposed for other similar derivatives of cotarnine and is, therefore, to be preferred to ' B ', notwithstanding the rather unstable character of this derivative and the ease with which it breaks up into its components on heating its solutions   It may be mentioned that, as far as can be ascertained, the only reference to a compound of cotarnine and urea is to be found in a German patent of Knoll & Co (1911) but no details are given therein with regard to its preparation and properties

Preparation and properties of anhydrocotarnine-carbamide —Pure cotarnine (10 grammes) is dissolved in absolute alcohol (60 c c) by warming, and a solution of urea (2 8 grammes) in the same solvent (50 c c) added, and the clear mixture heated in a half litre round-bottomed flask on the water-bath to a temperature not exceeding 45°C for half an hour   On cooling, the urea derivative separated almost immediately as colourless prismatic crystals which were collected after two hours, washed twice with cold alcohol (20 c c) to remove any unchanged cotarnine, dried in a vacuum over sulphuric acid and weighed   The compound prepared in this way formed pure white crystals weighing 11 grammes approximately   It changed colour at 160°C , and melted sharply at 182°C   The following results were obtained from combustion —

0 1736 gramme gave 0 3516 gramme of CO_2 and 0 1010 gramme of H_2O
Found   C, 55 3 per cent , H, 6 3 per cent ,
$C_{13}H_{17}O_4N_3$ requires C, 55 9 per cent , H, 6 1 per cent

The alcoholic filtrate from the crystals was concentrated but no further crops of crystalline material were obtained, showing that the precipitation had been almost quantitative

The substance is insoluble in cold alcohol, ether and benzene   It dissolves very sparingly in cold water but more readily in hot water and hot alcohol   The latter solvents could not be used, however, for recrystallization as the compound underwent considerable decomposition during the process of heating the solutions   It dissolves instantly in cold dilute hydrochloric acid to a pale yellow solution and was recovered unchanged on basification after an hour with cold dilute caustic soda   The precipitation did not, however, occur immediately, but slowly the solution turned milky and then crystals of the original substance (M P 182°C) separated out   When an aqueous solution of the compound was heated it became turbid due to the formation of free cotarnine which was identified as the picrate (fine needles, M P 143°C) A cold aqueous solution of the substance is found to have a pH ranging between 7 6 and 7 8, i e , it is definitely alkaline.

Pharmacological action

External action —The action of the drug on bacteria was studied by the Rideal-Walker method   This was kindly done for us at the King Institute, Guindy, by the courtesy of the Director, Lieut -Colonel H H King, i m s   The phenol-coefficient was found to be 4, showing that anhydrocotarnine-urea has some antiseptic value No irritant action is noticed on the skin or mucous membrane   A five per cent solution dropped into the eye of a rabbit does not produce anæsthesia   Subcutaneous injection does not set up any marked congestion, œdema or necrosis of the tissues

On the alimentary system —Anhydrocotarnine-urea interferes with peptic digestion *in vitro* in concentrations of 1 in 5,000 and more   In greater dilutions no appreciable effect is seen   Injections of 2 mg to 10 mg cause increased motility of both stomach and intestines of anæsthetized cats (Graph, fig c)   The stomach movements were recorded by means of the balloon method, the balloon at the end of a rubber-catheter being introduced through the mouth   The movements of the gut were recorded by means of Jackson's enterograph   The tone of the intestines is greatly increased but the effect is not lasting   Vagal division does not influence this result but after atropinization no more stimulation occurs, showing that the drug stimulates the parasympathetic nerve-endings in the gut   Cotarnine also acts similarly   But narcotine produces only inhibition

On circulation —Intravenous injections of 2 mg or more to anæsthetized cats cause a transient fall of blood-pressure   This fall occurs even after vagal division and atropinization and also in decerebrated animals   The volumes of the spleen and intestines are markedly increased (*vide* Graph, figs a and b)   The kidney volume and that of the hind leg show a definite decrease   Perfusion of the blood vessels of a pithed frog shows a quickening in the rate of outflow through the inferior vena cava lasting for about 2 minutes   Myocardiograph records on anæsthetized cats show a definite stimulation of the auricles with doses of 5 mg   This effect is abolished after paralysis of the vagal nerve-terminations with atropine   The apparent stimulation of the auricle is, therefore, ascribable to the depression of the vagal nerve-endings in the auricle   This effect is similar to that of some of the

cinchona alkaloids   On the ventricle the effect is inconstant   Sometimes a stim·ulation is noticed which is absent in other cases   On the isolated kitten's heart no stimulation is observed   Dilutions of 1 in 100,000 produce no effect while 1 in 10,000 tend to slow and steady the heart-beats   Higher concentrations slow and depress the heart   Dilutions of 1 in 12,000 cause a temporary depression of the isolated frog's heart   Bigger doses increase the depression

The drug, therefore, appears to have no marked effect on the heart, only depressing the vagus somewhat with smaller doses   A total of about 60 mg produced no appreciable depression of the heart in intact animals   The fall of blood-pressure seems to be mainly due to the dilatation of the blood vessels of the splanchnic area

When an intravenous injection of adrenaline is given to anæsthetized cats subsequent to a similar injection of anhydrocotarnine-carbamide, it is invariably noticed that the normal blood-pressure curve of adrenaline is completely changed   Instead of a sharp rise and a rapid fall to the normal, the rise of blood-pressure is sustained for a much longer period   In figs *e, f,* and *g* of the Graph it is observed that instead of recovery occurring in $1\frac{1}{2}$ minutes, the rise of blood-pressure is continued beyond $3\frac{1}{2}$ minutes   In some cases this effect lasted for even 6 minutes   When injection of both adrenaline and the drug is given together a similar phenomenon is noticed but the rise is not so high as when adrenaline alone is injected   This appears to be an example of synergism, the mode of action of which is not clear to us Sollmann states that the action of adrenaline is altered by conditions including other drugs and the changes are often more than additive   The modification of the blood-pressure reactions is said to be especially characteristic   Cotarnine-hydrochloride also is seen to act similarly

On respiration —Intravenous injection of this compound to anæsthetized cats invariably produces spasmodic breathing, in some cases leading to asphyxia   In both cases the movements are exaggerated, which might mean either a stimulation of respiration or spasmodic breathing caused by bronchial constriction   In certain other cases the movements became very shallow until asphyxia developed, perhaps due to a central depression   In order to make sure of the exact mode of action, lung volume was recorded in spinal animals   Instead of the plethysmograph described by Jackson which we found to be too large for our cats, we used a glass-cardiometer An opening was made into the chest of a spinal cat large enough to admit the circular rim of the cardiometer   The chest cavity was made airtight by suitable sutures A T-shaped cannula was connected to the cardiometer   One limb of this cannula was led by means of pressure-tubing to a volume recorder   To the other limb a short piece of rubber-tubing with a clamp on, was attached to serve as a by-pass Artificial respiration was given through the tracheal cannula   Changes in the calibre of the bronchi induced alteration in the expansion of the lungs which was transmitted to the volume recorder   An increase in the amplitude meant bronchial dilatation and a decrease, constriction   In this manner it was found that anhydrocotarnine-carbamide causes a definite contraction of the bronchial muscle Hence, the spasmodic breathing and asphyxia caused by the drug is due to bronchial constriction by peripheral action, in addition to a direct depression of the respiratory centre

GRAPH

(a) Lung Volume
    Intestine Volume
    BP
    ↑ cut 2.05 kilo urethan.
    anhydro-cotarn-urea 10mg
    8.9.33

(b) Spleen Vol.
    Anhyd-cotar-urea 2.5mg
    BP
    ↑ cut 1.95 kilo urethan 2.3.35

(c) Stomach movement
    BT
    ↑ anhyd-cotarnphre 5mg
    cat 2 kilo

(d) Respirations 2.2.1-34
    ↑ 1 in 100,000 Anhydrocotarn urea

(e) Adrenaline 0.05 mg.
    (after anhyd-cotarn-urea)
    T 6 sec

(f) 1'   2'   2'30   3'   3'30
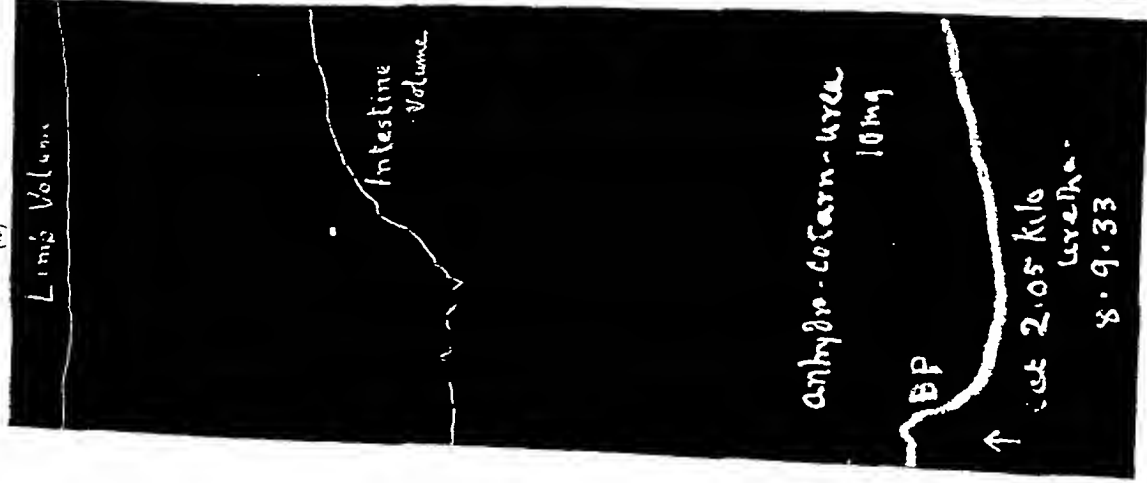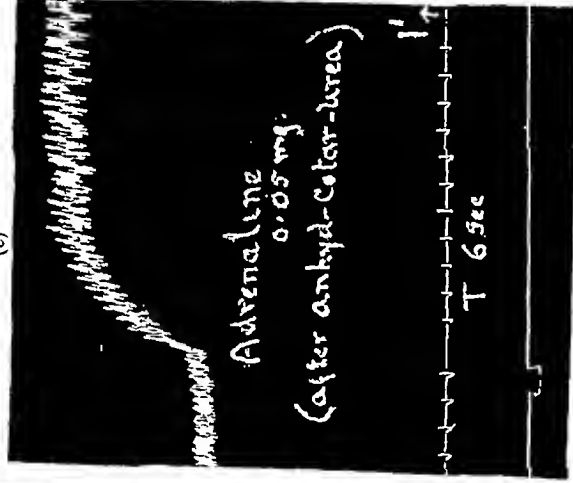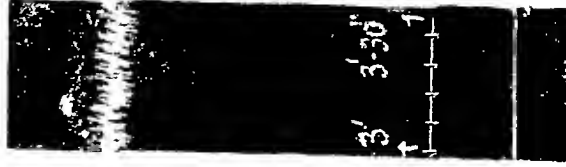
*On the central nervous system —*Injections of the drug were given into the lymph sac of frogs Ten mg and more first cause sluggishness of movements followed later by narcosis The postural reflex is lost before narcosis is produced The narcosis is so deep that the animal is insensitive to skin incisions Respiration stops quite early although the heart functions for hours afterwards As high a dose as 1 g per kilo of body-weight did not cause death or stoppage of the heart Frogs appear to be particularly resistant to this drug Subcutaneous injections of 300 mg per kilo induced no narcosis in rabbits nor was there any excitement Two hundred and fifty mg given to a cat had no appreciable effect

*On peripheral nerves —*A five per cent solution of the drug causes a block in the conduction of motor impulses along the nerve of a muscle-nerve preparation When the muscle is bathed in the solution it did not respond to stimulation of the nerve although direct stimulation of the muscle elicited the normal contraction, showing that the nerve-endings are also paralysed An increased irritability of the nerve is always noticed in the earlier stages

*On uterus —*Intravenous injections of 5 mg to anæsthetized cats lead to an increased tone of the uterine muscle as recorded by Jackson's enterograph This effect is temporary and comes on after a delay of 2 to $2\frac{1}{2}$ minutes A dilution as high as 1 in 200,000 causes an increase in the tone of the isolated uterus of a guinea-pig With 1 in 100,000 dilution the tone is markedly increased but this effect is not permanent (Graph, fig *d*) A subcutaneous injection of 250 mg was given to a cat in an advanced stage of pregnancy but nothing happened

*On temperature —*Kilham's method was followed to ascertain if this drug has any anti-pyretic action A dead emulsion of *B coli* prepared according to directions was given to raise the temperature in rabbits Doses ranging from 50 mg to 300 mg per kilo were given but had no effect in reducing the fever

*Toxicity —*The minimal lethal dose in rats when given subcutaneously is approximately 300 mg per kilo In fatal doses irregular breathing, and excitement towards the end, culminating in asphyxia and death are noticed

Summary and conclusions

Anhydrocotarnine-carbamide forms pure white prisms melting at 182°C It is a monacid base dissolving in cold dilute acids without any decomposition Its solutions, however, decompose on heating into cotarnine which can be easily recognized

The pharmacological action of this compound has been studied

Anhydrocotarnine-carbamide has a mild antiseptic action Its phenol-coefficient is 4 It causes incoordination and narcosis in frogs The respiration stops early but the heart continues to work for a long time It has an action on peripheral nerves, causing nerve block and paralysis of the motor end-plates No sensory paralysis is noticed

It depresses the circulation, mainly by dilatation of splanchnic vessels The cardiac action is not very pronounced

Plain muscles of the intestines, the bronchi and the uterus are markedly stimulated

No anti-pyretic action follows when the drug is injected subcutaneously into fevered rabbits

The minimum lethal dose of the drug to rats is about 300 mg per kilo

When administered with adrenaline or when the latter is given subsequent to this drug a synergistic action of the blood-pressure is observed

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STUDIES ON THE PROTEIN FRACTIONS OF BLOOD SERA

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Part I.

NORMAL AND FILARIAL BLOOD SERA

BY

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INTRODUCTION

IN a previous paper (Chopra, Chaudhury and Rao, 1930) it has been shown that the surface tension and buffer action of filariasis blood sera are somewhat diminished, while the density and the viscosity are not changed at all, in comparison with those of the blood sera of normal persons   Since there is a change in some of the physical properties, it is to be expected that there is also a variation in the composition of the blood   Boyd and Roy (1930) have reported a distinct increase in the cholesterol content in the blood serum of filariasis patients   Stenhouse (1925) also observed a superabundance of this lipoid body in the fresh lymph from filarial patients   But there is no information as to the increase or decrease of individual protein fractions or of the total proteins in the blood serum from such cases

The present work was, therefore, undertaken to obtain this information and to correlate it, if possible, with other physical properties observed in such cases

EXPERIMENTAL PROCEDURE

(*a*) *Estimation of the proteins and the globulin to albumin ratio* —The method used by us was essentially the micro refractometric method of Robertson (1915) as has been used by various investigators for this purpose (Rowe, 1917 , Righetti, 1916 , Hurwitz and Meyer, 1916 , Schmidt, 1917 , Basilico, 1926 , etc)   Estimations were made with the help of a Dipping Refractometer (Zeiss) with

the auxiliary prism and the metal-beaker arrangement, since the sera available were always too small for the glass beakers used with this apparatus

A saturated solution of ammonium sulphate at 21 5°C containing 53 5 g of ammonium sulphate per 100 c c of the solution was used as the precipitating electrolyte throughout the course of the experiments This temperature was chosen since the work had to be carried out in part at that temperature and solutions saturated at a higher temperature would on cooling release the excess of the salt A sample of 33 per cent and another of 25 per cent saturated solution of ammonium sulphate were prepared from this and were kept as stock solutions Refractive indices of these solutions were determined at different temperatures and the values represented in curves, from which by extrapolation, the refractive indices at any temperature for these solutions could be obtained Similarly a 0 02 N acetic acid was prepared from a sample of 0 04 N acid and its refractive indices extrapolated for different temperatures

Experiments were performed at the room temperatures which varied from 22°C to 26°C The fraction precipitated by 50 per cent saturation is taken as the total globulin, while the amount separated out with 33 per cent saturation is regarded as the euglobulin fraction and the difference between the percentages of these two is the percentage of the pseudoglobulin

The protein fractions in blood sera were determined after these preliminary operations Special precautions were used to clean all test tubes, pipettes, etc, with potassium dichromate sulphuric acid mixture before each experiment, and the data so obtained are reproducible within the limits of experimental errors as pointed out by Robertson, as will be evident from Table I in which are shown two results obtained under identical conditions with the same normal serum —

TABLE I

Nature of serum	Total proteins, g per cent *	Albumin, g per cent *	Pseudoglo-bulin, g per cent *	Euglobulin, g per cent *	Globulin albumin ratio
Acute filarial lymphan gitis	8 97	4 68	3 89	0 40	0 920
	8 99	4 62	3 99	0 38	0 922
Normal human serum	7 62	4 70	2 70	0 22	0 621
	7 61	4 65	2 72	0 23	0 635
Normal human serum	7 45	4 56	2 69	0 20	0 634
	7 31	4 56	2 65	0 15	0 620

* g per cent signifies the number of grammes per 100 c c of serum

(b) Surface tension was determined by the du Nouy's apparatus in the way indicated by Chopra and Chaudhury (1928) Care was taken to determine the true static surface tension and for this reason the readings were always taken where the string separated from the serum in 20 minutes' time

(c) Relative viscosity was measured by means of a micro viscometer of the Ostwald type The apparatus was thoroughly cleansed by potassium dichromate sulphuric acid mixture before investigation of each serum

RESULTS AND DISCUSSION

Our work on normal cases which have been collected for comparison with the corresponding data for filariasis blood sera, constitute *Part A* of the present paper

Data for filariasis cases have been incorporated in *Part B*

Part A.

Lloyd and Paul (1928,1929) in connection with their work with kala azar cases have determined the different protein fractions as well as the globulin to albumin ratio for normal Indian blood sera But in the two papers in which their results were published we find two different average values for the globulin to albumin ratio, the actual values being 0 662 and 0 638 respectively Linder, Lundsgaard and van Slyke (1924) working with a different chemical method obtained 0 635 as the ratio for the average normal European In the literature, however, one can find different values for normal Europeans recorded by different investigators For this reason we thought it advisable to determine for ourselves these figures for normal Indians The results are given in Table II Surface tension, relative viscosity, pH and buffer action for some of these cases have been incidentally determined and they are found to be in agreement with those published in previous papers by Chopra and Chaudhury

From Table II it will be evident that for normal Indians the globulin to albumin ratio varies from 0 588 to 0 643, the average of the eleven cases observed being 0 611 The percentages of albumin vary from 4 30 g to 4 98 g per 100 c c of serum, the average being 4 61 g, while the pseudoglobulin and euglobulin vary from 2 40 g to 2 90 g and 0 13 g to 0 27 g per 100 c c respectively The total proteins, however, vary from 6 94 g to 7 99 g the average being 7 44 g These are in agreement with such data given by Lloyd and Paul (1928,1929) within the limits of experimental error —

TABLE II

Serum from normal Indians

Number	Total proteins in g per cent *	Albumin in g per cent *	Pseudoglobulin in g per cent *	Euglobulin in g per cent *	Globulin albumin ratio
1	7 99	4 98	2 80	0 21	0 601
2	6 95	4 30	2 40	0 25	0 603
3	7 62	4 70	2 70	0 22	0 622
4	7 54	4 69	2 71	0 14	0 601
5	7 07	4 33	2 61	0 13	0 630
6	7 65	4 80	2 71	0 14	0 591
7	7 86	4 70	2 91	0 25	0 643
8	7 10	4 50	2 43	0 27	0 600
9	7 73	4 87	2 67	0 19	0 588
10	7 45	4 56	2 69	0 20	0 634
11	6 94	4 30	2 50	0 14	0 613
Average	7 44	4 61	2 65	0 18	0 611

* g per cent signifies the number of grammes per 100 c c of serum

Relative viscosity, surface tension, pH and buffer action of 1 to 5 of the above cases are given in the following table —

TABLE III

Serum from normal Indians

Number	Relative viscosity	Surface tension in dynes	pH *	BUFFER ACTION	
				0 6 c c serum and 0 4 c c 0 01N HCl	0 4 c c serum and 0 6 c c 0 01N HCl
1	1 71	59 0	7 53	7 21	6 87
2	1 57	62 0	7 22	6 98	6 88
3	1 63	60 4	7 54	7 13	6 83
4	1 60	58 7	7 54	7 13	6 83
5	1 56	60 4	7 59	7 29	6 97
Average	1 60	60 1	7 48	7 14	6 88

* The pH was calculated from the E M F of the chain Hg/Hg_2Cl_2, sat KCl/sat KCl/ hydroquinhydrone in serum/Pt

The euglobulin among the different fractions shows the greatest variations, the value being in some cases 100 per cent greater than in others, while the average value 0 18 is very near to those determined by different investigators   It is evident from the above table that the individual fractions and the total proteins exhibit a much greater variation than the globulin albumin ratio, which is more or less constant within certain limits, while the variations in the euglobulin fractions are the most prominent   Excepting the euglobulin fraction, the variations in other fractions including the globulin albumin ratio as found by us can be accounted for as due to the individual variations in different human beings *

From the literature it is evident that many investigators have carried out their experiments on precipitation and estimation of refractive indices at their room temperature which of course varies in different countries   Here in India the room temperature varies very widely in different seasons but in any particular season it is found to vary within 2 to 4 degrees at a fixed time of the day   In order, therefore, to find out whether the values for the different protein fractions as well as for the globulin albumin ratio as determined by different investigators are appreciably influenced by factors such as   (a) temperature, (b) concentration of the precipitating electrolyte and (c) other actual experimental conditions, we incidentally performed a few experiments in that direction   It is, however, to be expected that

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* Salvesen (1926) from 42 determinations in 32 normal men and women showed an average of total proteins in blood plasma to be 7 0 per cent with variations between 6 34 to 7 96   Repeated determinations in the same individual, however, show considerable variations in the total proteins as well as albumin globulin ratio (average 1 62, minimum 1 26 and maximum 2 0) which corresponds to a albumin globulin ratio of an average of 0 617, maximum 0 793 and minimum 0 500

these factors should not have any great effect upon lyophillic colloids like the protein solutions but our object is to find out whether the changes caused by these factors fall within the limits of individual variations We do not claim that our work in this direction has been thorough and exhaustive but the few cases that we studied, at least, indicate the direction and magnitude of these variations in normal cases

(a) *Temperature* —In order to ascertain whether this brings about any difference in the data obtained in such experiments we determined by the above method the proteins of the sample of serum at two widely different temperatures, viz , 29°C and 12 5°C the results of which are given in the table below The globulin to albumin ratio shows a small difference —

<div align="center">

TABLE IV

Serum from normal Indians

</div>

Temperature in degrees C	Total proteins, g per cent *	Albumin, g per cent *	Globulin, g per cent *	Euglobulin, g per cent *	Globulin albumin ratio
29	8 32	5 25	2 99	0 23	0 620
12 5	8 32	5 36	2 66	0 30	0 532

<div align="center">

* g per cent signifies the number of grammes per 100 c c of serum

</div>

The influence of temperature is thus seen to be considerable for the range of temperature studied The temperature coefficient, however, seems to be very small and hence it is to be expected that a small difference of temperature of 2°C to 4°C would have no appreciable influence on the results obtained, while a larger difference is likely to bring about a comparatively wider variation in the results

(b) *Concentration of the precipitating electrolyte* —The temperature influences the concentration of the precipitating electrolyte since in these experiments a saturated solution of ammonium sulphate is generally used, but this can be avoided by using a definite concentration of the electrolyte throughout We however, examined the influence of the concentration of the precipitating electrolyte (viz , ammonium sulphate) by treating two different samples of sera with varying degrees of saturation with ammonium sulphate and determined the amount of proteins that still remained in the solution after each precipitation The data so obtained are plotted in Curves I and II In Curve I the concentration was varied from 20 to 55 per cent of saturation with ammonium sulphate, while in Curve II it was varied from 20 to 70 per cent In both of them the run of the curves is similar for the stretch of the curves which is common In both the cases we find that within 30 to 40 per cent of saturation, the curves have a small slope, while beyond 40 per cent the run of the curves is evidently different being steeper than the previous portion It is quite probable that the curves beyond 40 per cent represent the precipitation of a different protein fraction The region between 30 and 40 per cent may be due to the constituent euglobulin But possibly pure euglobulin seldom comes out under such circumstances as the zones of precipitation in the case of protein solutions, as is well known, are not sharp, in consequence of which the precipitate may be found contaminated with some pseudoglobulin as well

Beyond 45 per cent saturation with ammonium sulphate, Curve II again runs steeper still up to 50 per cent and then becomes flat again This latter part of the curve may be regarded as the transi tional portion wherein the pseudoglobulin is separated out together with the albumin The curve, however, is not perfectly horizontal in this region as it ought to be in such cases but a little sloping showing thereby that all the globulins have not been precipitated at 50 per cent saturation and a part of this remains behind and is precipitated up to 60 per cent saturation, from where a steeper part indicates the separation of a different fraction The slope of this region between 50 and 60 per cent saturation corroborates also the observations of previous workers in this line that this region is not entirely free from precipitation as has been suggested by Hofmeister (1888) This can also be demonstrated by adding a drop or two of saturated ammonium sulphate solution to a definite portion of the clear centrifugate obtained after treatment with these percentages of saturation with ammonium sulphate when a slight turbidity appears in each case which is very prominent with 45 per cent saturation and comparatively slight with 55 per cent
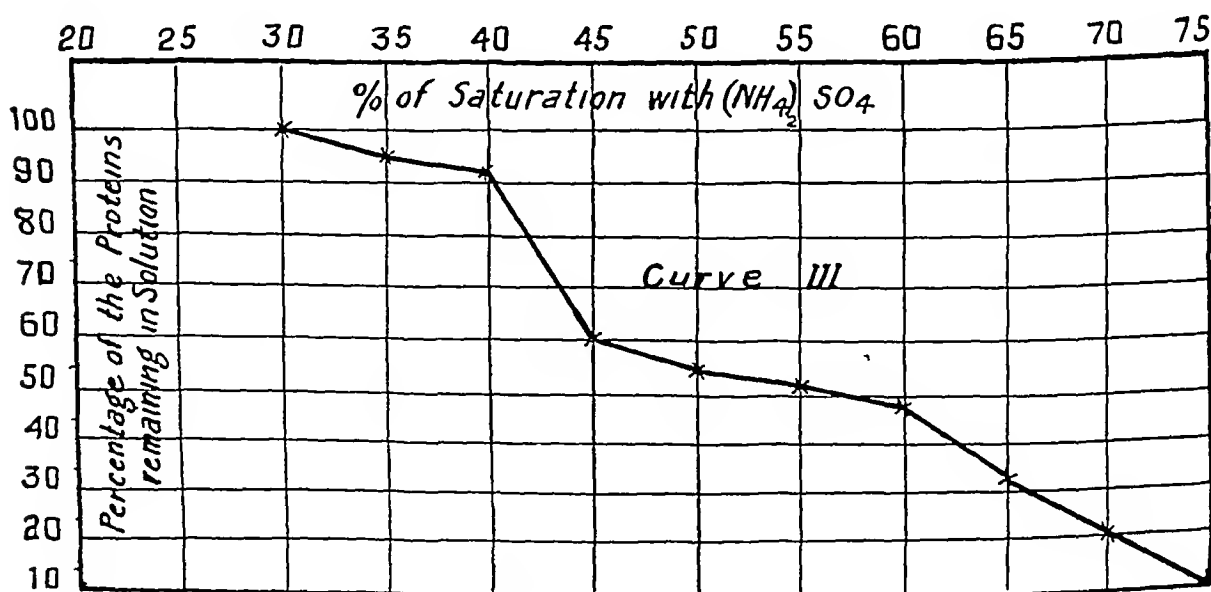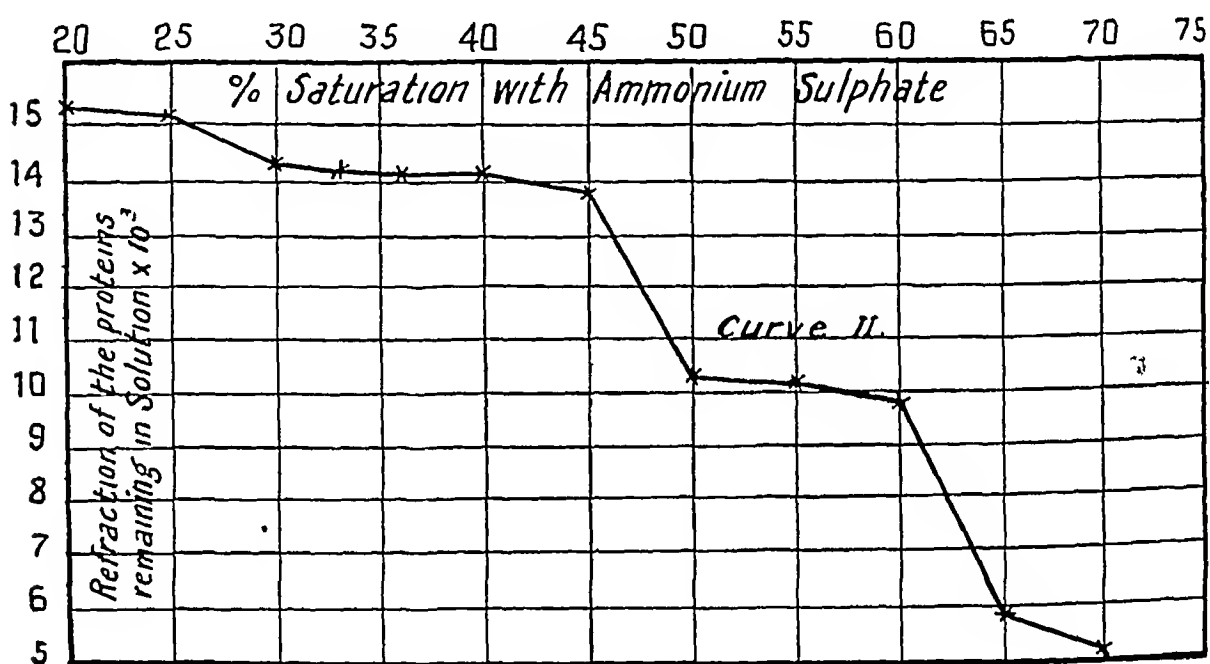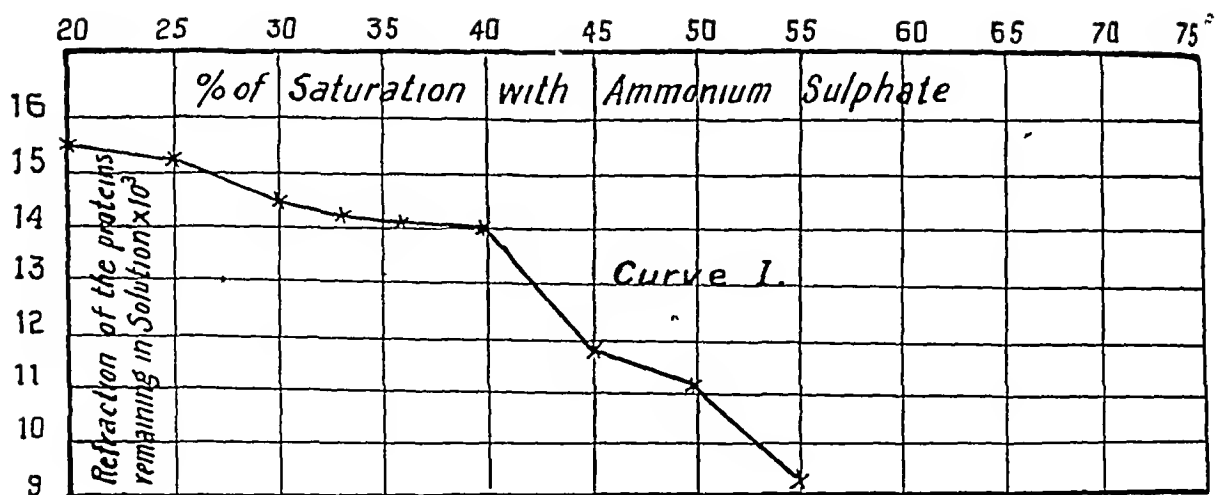
Rusczsinsky's precipitation curve for normal sera

The curves obtained by us are broadly similar in nature to those obtained by Rusczsinsky (1924) who working with normal serum by analytical methods obtained curves given in Curve III

The significance of these curves is evident They indicate that the precipitation regions of the different protein fractions are overlapping one another due to which repeated fractionation will be necessary to separate any of the fractions completely and in a comparatively pure state (Haslam, 1905) Hence in the fractionation process as used in the micro refractometric method the constituent proteins seldom separate out completely and in a pure state which may be found in one or two isolated cases only This obviously introduces an error though small, which varies differently with different individuals according to the amount of the total protein content in the serum

(c) *Changes in the experimental conditions* —In our investigations on the normal and filariasis blood sera we strictly followed the orthodox method of Robertson (1915) the only modification being that the amount of euglobulin was determined not by treatment of the serum with carbon dioxide gas but by fractionating with 33 per cent saturation with ammonium sulphate But we studied the changes brought about in the method of precipitation in the following ways —

1 In Robertson's method the proteins are always precipitated from serum by adding serum to ammonium sulphate and not by adding ammonium sulphate to serum, since in the latter case some albuminous bodies are likely to come out, due to which the results are irregular We thought it necessary to study this point since from the literature it is not clear what exact method of procedure is followed by different investigators so far as the above point is concerned The results for a few normal cases only have been given in the following table —

<div align="center">

TABLE V

Serum from normal Indians
</div>

Number	Tempera ture in degrees C	Conditions of experiment *	Total protein, g per cent †	Albumin, g per cent †	Total globulin, g per cent †	Euglobulin, g per cent †	Globulin albumin ratio
1	12 5	1	7 43	4 87	2 56	0 29	0 53
		2		4 90	2 53	0 26	0 52
2	12 5	1	6 71	4 23	2 47	0 21	0 58
		2		4 25	2 46	0 20	0 55
3	17 5	1	7 80	4 93	2 87	0 26	0 58
		2		4 98	2 82	0 24	0 57

* Condition 1—Serum was added to ammonium sulphate
   „   2—Ammonium sulphate was added to the serum
† g per cent signifies the number of grammes per 100 c c of serum

It is evident that the differences as obtained in these cases are small and need not be taken into account in consideration of the variations observed in individual cases

2 The next point we studied is given below In the refractometric method for the estimation of proteins we usually treat the serum with 50 per cent saturation with ammonium sulphate But the refractive index of the clear liquid obtained after the removal of the precipitates, which is supposed to contain the albumin and the non proteins of the serum, is not compared with that of a 50 per cent saturated solution of ammonium sulphate for the determination of albumin thereof, because according to Robertson (1912) the protein solutions in 50 per cent saturated ammonium sulpbate solution show abnormal refractive indices Hence the clear solution so obtained is diluted twice with water and the albumin calculated by a comparison with 25 per cent ammonium sulphate solution This latter procedure although followed by most of the investigators is not strictly adhered to by many as appears

from the literature   For this reason we studied this condition and the results so obtained have been given in the following table —

TABLE VI

Serum from normal persons

Number	Temperature in degrees C	ALBUMIN IN G PER CENT	
		With ½ saturated ammonium sulphate	With ⅓ saturated ammonium sulphate
1	12 5	5 88	4 87
2	12 5	4 55	4 23
3	17 5	5 46	4 93

Here the amounts obtained by these procedures differ very markedly   The amounts calculated with 50 per cent saturated ammonium sulphate solution are always a little higher than in the other case   It is quite clear from this that the values of globulin to albumin ratio calculated with these different data for albumin will also differ   Similar results have also been obtained with a few other samples of sera (not given here) and the data for albumin were consistently found greater in one case than in the other   This fact must, therefore, be borne in mind when dealing with such cases

3   The third point that we investigated in this connection was to determine whether in the case of 33 per cent saturated ammonium sulphate solution there is any difference in the results obtained by using the whole serum as a diluent for the saturated ammonium sulphate solution and those obtained by using a mixture of water and serum in equal volumes as a diluent for the saturated ammonium sulphate solution   The reversibility of the process of the protein precipitations, however, leads us to expect no changes in the result, if the final conditions in both the cases are the same   But dilution with water in the case of the whole serum especially in some pathological conditions like kala azar, etc , precipitates the euglobulin thereof   Dilution brings about a diminution in the concentration of the serum electrolytes and also changes the hydrogen ion concentration of the serum although to a very small extent   These two factors have a precipitating influence upon the euglobulin present in the serum   Our results are given in Table VII —

TABLE VII

Serum from normal Indians

Number	Temperature in degrees C	EUGLOBULIN IN G PER CENT *	
		With whole serum as diluent	With a mixture of serum and water in equal volume as diluent
1	12 5	0 25	0 29
2	12 5	0 17	0 21
3	17 5	0 19	0 20

* g per cent signifies the number of grammes in 100 c c of serum

It is evident that results differ to a certain extent under the two conditions   It is quite likely that in such cases of reversible precipitation there is some lag of time to attain equilibrium in these

Part B

Protein fractions and the globulin albumin ratio were determined in the different conditions of filariasis, acute lymphangitis, and chyluria   Relative viscosity and surface tensions were also determined in these cases side by side The results are given in Table VIII —

TABLE VIII

Serum from filariasis blood

Number	Nature of the case	Total protein, per cent	Albumin, g per cent	Pseudoglobulin, per cent	Euglobulin, per cent	Globulin albu min ratio	S T in dynes	Relative visco sity
1	Acute filarial lymphan gitis	7 38	3 98	2 90	0 50	0 853		
2	,,	6 10	3 40	2 30	0 40	0 793	62 3	1 50
3	,,	7 65	4 30	3 20	0 15	0 799	60 9	1 60
4	,,	6 66	3 89	2 70	0 19	0 743	60 6	1 55
5	Elephantiasis and acute lymphangitis	7 80	4 40	3 10	0 30	0 773	57 3	1 56
6	Lymphangitis (acute)	9 06	4 68	3 98	0 40	0 936	57 3	1 60
7	,,	8 69	3 89	4 50	0 30	1 230	58 6	1 50
8	,,	8 50	4 71	3 23	0 56	0 804	56 6	1 70
9	,,	5 00	3 40	1 90	0 60	0 735	61 3	1 40
10	,,	7 00	3 80	2 70	0 50	0 842	57 8	1 65
11	,,	8 40	4 90	3 25	0 25	0 714	61 5	1 46
12	,,	9 08	4 70	4 03	0 35	0 931	57 5	1 67
13*	,,	4 80	2 00	1 80	1 00	1 200	58 1	1 50
14	,,	7 12	4 10	2 95	0 17	0 736	58 1	1 57
15	,,	7 15	3 45	3 45	0 25	1 072	58 0	1 43
16	,,	8 40	5 10	2 90	0 40	0 647	57 3	1 65
17	Chyluria	7 20	3 50	3 20	0 50	1 060	56 2	1 63
18	,,	5 30	2 80	2 02	0 48	0 892		
19	,,	8 23	3 96	4 27	0 28	1 124	57 9	1 65

Per cent or g per cent signifies the number of grammes per 100 c c of serum

* Hæmolysis took place to a considerable extent and hence the data are not reliable

Here we find an increase in the globulin albumin ratio in all cases, the increase being slight in some cases and marked in others   In less acute cases (from Nos 9 to 16) we find the ratio to vary between 0 647 to 0 736, whereas in the acute cases of filarial lymphangitis and also where elephantiasis is associated with this, the ratio is found to vary from 0 773 to 0 936 (Nos 1 to 8) the value being as much as 1 072 in one case only   In the three cases of chyluria studied herein, the ratio was found to be much greater varying from 0 892 to 1 124   In case No 16 which was reported to be recovering from the disease value of the ratio was 0 647, very near to the average normal figure, viz , 0 611

Total proteins were within the normal range excepting two cases where the values are as high as 9 06 and 9 08 respectively and two more cases where the values were considerably less than the normal figures (viz , 5 00 and 5 30)   In all cases we observed a slight increase in the amount of total globulin, the individual consti tuents, viz , pseudo- and euglobulins exhibiting an increase in most of the cases, while in a very few the value is practically normal   But whether such values are really normal in these individual cases cannot, however, be definitely ascertained for want of normal figures for the self-same persons   The albumin fractions in most of the cases seem to be diminished to a certain extent   The pseudoglobulins in the majority of the cases studied, showed an increase as compared with the normal figures   But as regards the euglobulin fraction we find the range of its variation very wide from 0 15 to 0 60 per cent

Gardner and Gainsborough (1927) have shown in cases of nephritis, especially of the parenchymatous type, that the blood cholesterol shows a definite increase and those protein fractions which are more associated with cholesterol, namely the globulins, especially the euglobulins, also increase in such cases   Hence the globulin to albumin ratio is altered in the corresponding direction   In filariasis cases such correlation between the cholesterol and the euglobulin percentages does not seem to be impossible in view of the work of Boyd and Roy (*loc cit*) on the cholesterol content of filarial blood sera and our data showing the increase of euglobulin in such cases

But in the light of recent work of Schretter (1926) on the refractivities of albumin and globulin in pathological sera there appears considerable doubt as to whether the proteins in different pathological sera are identical in their chemical nature and constitution   Thus, euglobulin from kala-azar blood sera, as shown in previous papers (Chopra and Chaudhury, 1929, 1932 , Chopra, Chaudhury and De, 1931), have properties and behaviour quite different from those of the euglobulin obtained from normal blood sera and the probability of the existence of a new protein in such cases has been pointed out   It is very likely that in a similar way the protein fractions under other pathological cases also may lose their identity as such and become converted into slightly different new proteins due to changes in their internal structures   The problem, however, awaits a thorough chemical examination of the different proteins with respect to their constitution and properties, as well as a simultaneous estimation by the refractometric method   Unless this is done it is not, *a priori*, possible to discuss how these individual fractions really increase in such cases

The viscosity determinations in these cases show, however, very little devia tions from the normal (*cf* Table VIII and also Chopra, Chaudhury and Rao, 1930)

while the surface tension is slightly diminished   It appears from the figures observed that both viscosity and surface tension depend not only upon the amount of the total proteins in solution but also upon the relative amounts of the individual fractions   In almost all the filarial cases studied herein we find a diminution in the percentages of albumin while those of the euglobulin content increase to a certain extent   Whether these two have got a compensating influence upon the viscosity or whether this is due to the change in any other blood constituents cannot be definitely ascertained until a thorough examination of all the constituents be made in such cases

SUMMARY AND CONCLUSIONS

The protein fractions have been estimated for normal sera under different conditions and it has been shown that different observers get different values for the globulin albumin ratio possibly due to the different conditions of experiment viz, temperature, concentration of the electrolyte and the actual conditions of precipitation

Following Robertson's method it has been found that the globulin to albumin ratio agrees fairly well with those obtained by Lloyd and Paul (1929)

The total globulin, especially euglobulin, in filariasis patients increases while albumin decreases to a certain extent in consequence of which the globulin to albumin ratio is found to increase

The physical properties such as surface tension, viscosity and buffer action of sera from filarial blood change very little if at all   What this is due to cannot be definitely ascertained, until all the serum constituents be determined in such cases

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ANTHELMINTIC PROPERTIES OF *VERNONIA ANTHELMINTICA* WILLD (SYN *SERRATULA ANTHELMINTICA*)

BY

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THIS plant belongs to the natural order *Compositæ* and is found throughout India, from Kashmir to Ceylon   It is a tall, robust, leafy annual with stems about 2 to 3 feet long   In Hindi it is known as *Somraj, Bakchi*, in Bengali *Somraj*, and in Sanskrit as *Vakuchi* or *Somaraja*   The achenes (seeds), which are chiefly used in medicine, are about 3/16 inch long, of a dark-brown colour, covered with whitish scattered hairs, cylindrical, tapering towards the base, marked with about 10 longitudinal ridges, and crowned with a circle of short brown scales   The taste is nauseous and bitter

The seeds have been used in the treatment of leucoderma and psoriasis and are considered to have powerful anthelmintic properties, especially when combined with other remedies (Dutt)   An infusion made from 20 to 60 grams of powdered seeds is said to be an effective anthelmintic against ascarids   The drug has also been regarded as a valuable tonic and stomachic, and diuretic properties are also assigned to it   The seeds are given internally in leprosy and form part of preparations such as pastes, oils, etc , used as external applications in leucoderma, psoriasis and other skin diseases   In the Nighantas they are described as sweet, pungent, digestive, bitter, alterative, astringent, cold, dry and antiphlegmatic and are recommended for cough, fever and intestinal worms   A decoction made from roots and leaves with coumiss and butter milk is used in Malabar in diarrhœa and dysentery   In Travancore, the bruised seeds ground up in a paste are largely employed as a means of destroying pediculi   They are also given internally in anasarca and are

applied externally as plaster to abscesses　The seeds are considered in the Punjab to be a febrifuge

Dymock, Warden and Hooper (1891) record the following short description of the results of chemical analysis　'The alcoholic extract contains a large amount of an amber coloured oil soluble in petroleum ether, as well as resins　By agitation of the alkaline alcoholic extract with ether a somewhat bitter extract was obtained, which, besides containing resins, afforded evidence of the presence of an alkaloid, which gave reactions with the usual alkaloidal reagents, but which afforded no special colour reactions　We have provisionally called this principle Vernonine'　A more recent work (Kirtikar and Basu, 1918) gives the following description of the chemical examination of the oil　'The seeds on extraction with ether yielded 18 25 per cent of a dark brown coloured and strong smelling oil with some resinous matter　The expressed oil is of light yellow colour and very viscid, it deposits "Stearin" on standing　Physical and chemical characteristics of fat　sp gr at 100°C 0 9168, acid value 58 2, saponification value 202 88, Reichert Meissl value 7 88, iodine value 71 0, butyro refractometer at 25°C 75, fatty acids 91 6 per cent, melting point 35 6°C, iodine value 73 4, neutralization value 195 1, mean molecular weight 287 4' (A K Menon)

No recent work appears to have been undertaken with a view to study its chemical composition and therapeutic action　As the drug is largely used in Hindu medicine we took up its study, the results of which are recorded in this paper

<div align="center">CHEMICAL EXAMINATION</div>

The powdered seeds were extracted successively with petroleum ether, chloroform and alcohol, the solvents were removed, the residue dried and weighed　Petroleum ether extracted 18 4 per cent, chloroform extracted 1 2 per cent and alcohol extracted 13 8 per cent of the weight of the seeds　The petroleum ether extract consisted mainly of a fixed oil　It also contained a very small amount of an essential oil as described later　The chloroform extract was a greenish mass, containing all the bitter substance of the seeds　It was soluble in alcohol but insoluble in ether and cold water　It was very sparingly soluble in hot water, the solution being very bitter to taste　It gave no reaction for alkaloids　The alcoholic extract was extracted with water and filtered　The acid aqueous filtrate gave tests for tannins but not for alkaloids　The residue consisted of resins and phlobaphenes

For a more detailed examination, 3 kilos of the whole seeds were exhausted with 90 per cent hot alcohol　The alcohol was distilled off and the residue distilled in steam　The distillate was extracted with ether and the ethereal solution on evaporation gave 0 02 per cent of an essential oil　The oil had a pleasant odour and a slight cooling, peppermint like taste and a portion crystallized out in needles　The quantity obtained, however, was too small for further examination or identification　The residue obtained after steam distillation was allowed to cool when it separated out into three layers, an upper oily layer, an aqueous solution and a soft resinous solid　The oily layer was found to be the fixed oil already described　The aqueous layer was decanted off, the resin was washed several times with hot water and the washings added to the aqueous liquid　The aqueous liquid which was acid in reaction was extracted with ether and the ether evaporated off　The residue gave reactions for tannins　The aqueous solution was then made alkaline with ammonia and shaken first with ether and then with chloroform　Neither the ethereal extract nor the chloroform extract gave any appreciable residue and none gave any reactions for alkaloids　The aqueous solution was next treated with neutral lead acetate　The precipitate was filtered, washed, suspended in water and decomposed by H₂S　The filtrate thus obtained, freed from H₂S, gave reactions for tannins　The final filtrate from lead acetate precipitate gave nothing of a definite nature

The soft resinous matter was treated with petroleum ether to remove any adhering fixed oil　The solid residue consisted of some of the bitter substances, resins and phlobaphenes

We found that a light yellow very bitter substance could be obtained from the seeds when they were rubbed with the hands　On microscopic examination they appeared to be yellow globular bodies mounted on minute hairs which cover the whole surface of the ridges of the seeds　This yellow powder is very sparingly soluble in hot water and imparts to it a bitter taste and yellow coloration　Alcohol dissolves the yellow substance completely leaving only the cellulose matter of the hairs, etc　The alcoholic solution after evaporation leaves a yellowish varnish like resinous body nearly completely soluble in chloroform and precipitated by petroleum ether　In fact, it behaves exactly like the bitter substance isolated from an alcoholic extract of the seeds　Again, the bitter principles can be wholly, if not entirely, dissolved out by rectified spirit from the whole seeds without crushing them　It, therefore, appears that this substance is distributed mainly on the superficial glandular hairs found on the ridges of the seeds　We have also obtained a good yield of the bitter substance from the dusts, etc,

which the drug merchant throws away during winnowing the seeds to separate them from adhering earthy matter, etc It is quite clear that since the activity of the drug mainly depends on the bitter substance, as observed by clinical trials, much of the active principle may thus be lost through careless handling of the seeds and it was also found that fresh seeds in which these glandular hairs are rather elastic and pliable and which have not undergone much handling, are more bitter and hence efficacious than the older seeds in which these hairs become very brittle through drying and hence liable to be blown away by rough handling

Isolation and purification of the bitter substance —In order to isolate the bitter substance which is the main active constituent of the seeds, several kilograms of the seeds were extracted in a percolator in the cold with rectified spirit   The alcohol was distilled off and the residue repeatedly extracted with chloroform   The chloroform extracts were filtered, concentrated and the bitter substance precipitated with petroleum ether   The precipitate was dried and when powdered appeared as a greenish yellow powder   The yield of the crude bitter substance was about one per cent of the weight of the seeds It was further purified by repeating the process of extraction with dry chloroform, concentration and precipitation with dry petroleum ether   The bitter substance was finally obtained as a bright yellow amorphous powder   It melted with decomposition at about 95°C   It contained no nitrogen or sulphur   It was very slightly soluble in ammonium carbonate solution (10 per cent), somewhat soluble in 10 per cent Na_2CO_3 but more easily soluble in 10 per cent NaOH or KOH   From its solution in alkali it was precipitated by HCl   It was soluble in glacial acetic acid   The bitter substance thus appears to be of the nature of resin acids

Therapeutic properties —The diuretic as well as the antiseptic and stimulant effects of the seeds on the skin described in the Hindu medical literature are probably due to the small quantities of the essential oil and resins contained in the seeds , these, however, are not very marked   The chief use of the seeds is as anthelmintic and these properties were carefully tested by us in a series of patients   Not only were the powdered seeds tried, but we also tested the anthelmintic activity of the purified bitter principle isolated as well as other relevant fractions obtained during the course of analysis

The powdered seeds in doses varying from half to one drachm expelled ascaris in a number of cases but their vermifugal action was not so certain as of the drugs like santonin   Their action against oxyuris vermicularis was much more powerful, a large number of the worms being expelled after administration of the seeds

That the bitter substance contained in the seeds is the active anthelmintic principle, is shown by the fact that in a series of 25 cases in which it was tried in the Carmichael Hospital for Tropical Diseases, it proved effective in expelling ascaris in one patient and oxyuris in a large number of patients

The purified bitter substance was given at bed time either alone or combined with 3 to 5 grains of calomel, followed the next morning by a dose of magnesium sulphate   In 15 cases in this series in which oxyuris infection was diagnosed, 11 passed adult worms with the combined treatment   In some patients the drug had to be repeated 2 or 3 times before all the worms could be expelled   The drug is generally well tolerated and in only one patient in this series was vomiting produced, after its administration on two occasions   The drug had no effect whatever on the hookworm and tapeworms   It must be remembered that the oxyuris are easily expelled by such purgative drugs as calomel and salts, but the number expelled when these were combined with *Vernonia anthelmintica* was considerably larger Besides this in a number of cases so treated, the administration of carbon tetra-chloride afterwards expelled very few and sometimes no worms at all showing that the drug was effective   Some of the patients were followed up and on examination a few months later were found to be free from the worms as well as from the symptoms produced by this infection

SUMMARY AND CONCLUSIONS

The seeds of *Vernonia anthelmintica* contain —

(a) About 18 per cent of a fixed oil, (2) about 0 02 per cent of an essential oil, (3) a bitter principle, and (4) resin, tannins, phlobaphenes, etc

(b) Powdered seeds in doses of 30 to 60 grains have a weak vermifugal action against ascaris and a more powerful action against oxyuris   The bitter substance isolated, in doses of 3 to 10 grains, has a weak vermifugal action against ascaris and a decided action against oxyuris When combined with calomel and followed by magnesium sulphate the vermifugal action is considerably enhanced

(c) The drug has no action whatsoever against the hookworm and tape-worms

(d) The anthelmintic properties of the drug against ascaris and even against oxyuris are weaker and in no way comparable with some of the other compounds now in the British Pharmacopœia.

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STUDIES IN RAT LEPROSY.

BY

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PROGRESS in knowledge of human leprosy is exceedingly slow   It is extremely doubtful whether a culture of *M lepræ* has yet been obtained and there is available no susceptible animal for experimentation   Many leprologists have, therefore, turned to a study of rat leprosy as a possible source of indirect light on the problem of human leprosy   In this paper we make no attempt to undertake a thorough study of the subject   We deal mainly with certain aspects of rat leprosy which have been investigated in the Leprosy Department of the School of Tropical Medicine, Calcutta, during the years 1932-1933

A COMPARISON BETWEEN HUMAN AND RAT LEPROSY

Both diseases are caused by acid-fast bacilli belonging to the group *Mycobacteria* The two organisms are morphologically identical   In both cases it is doubtful whether culture on artificial media has been accomplished   The rat disease is probably transmitted from rat to rat by infection of wounds and abrasions, and the human disease is possibly transmitted in the same way   In our experience, all rats are susceptible to rat leprosy, the injection of even small doses of bacilli being followed by the development of the disease in practically every case   With human leprosy, however, there is considerable evidence to show that only a comparatively small proportion of human beings is susceptible and that children are more suscep- tible than adults

In both diseases there is a long latent period before the disease becomes apparent In human beings this period varies from a few months to many years and often the infection does not produce clinical manifestations at all   In the rat, if the infecting dose is small, the disease may never show itself outwardly, but only be detected at post-mortem examination after death from old age or intercurrent disease   If the infecting dose of bacilli is large, death usually occurs from genera- lized rat leprosy between six months and a year after infection

Human leprosy affects principally the nerves, the skin, and the reticulo endothelial system   Rat leprosy experimentally induced affects the skin at the site of injection and the nerves not at all, but principally the lymphatic glands, the reticulo-endothelial system, and the internal organs, particularly the liver and spleen   This matter is discussed more fully in the section on pathology.

BACTERIOLOGY

The relation between the two organisms M   lepræ *and* M   lepræ muris

Marchoux and Sorel (1913) considered that the two organisms have a relation-ship similar to that of the human and avian types of *M   tuberculosis*   This seems a reasonable opinion, but it has not been proved   Bayon (1914) considered the two organisms as closely related if not identical, and that rats injected with human leprosy organisms showed the same lesions as those injected with rat-leprosy orga-nisms   Walker and Sweeney (1929) considered that the two organisms are identical, but they quote no experimental evidence whatever in support of this opinion   Marchoux (1922) reported one case of a human being infected with *M. lepræ muris*   The lesions were different from those of ordinary human leprosy   Laigret (1932) injected a leprosy patient with *M   lepræ muris* with inconclusive results   We have injected many rats with an emulsion of bacilli made freshly from nodules of cases of human leprosy   In not a single rat, even after many months, have we seen the development of the progressive systemic infection which is typical of rat leprosy   We have, it is true, found acid-fast bacilli in the tissues for twelve months after the injection, but this alone does not indicate the existence of rat leprosy   This leads us to a matter which is of considerable importance and the lack of an understanding of which is leading to confusion in leprosy work

The effect of injection of acid-fast bacilli into animals

The injection of non-pathogenic acid-fast bacilli into rats produces the following effects   There is a swelling at the site of injection and swelling of the neighbouring lymphatic gland ,   the bacilli enter the blood stream and are deposited in the various organs of the body particularly the lungs, liver, spleen and lymphatic glands ,   here there is a cellular response to the bacilli, which are ingested by phagocytic cells   The bacilli gradually get fewer in number and finally disappear but often the bacilli can be detected for six and even for twelve months after injection

We have produced this effect in rats by injection of Hansen's bacillus, Kedrowsky's bacillus, Uchida's bacillus, Cilento's bacillus, and others   *Exactly the same effect can be produced by the injection of emulsions of acid-fast bacilli killed by heat in an autoclave (see* Plate   XI, figs   1 *and* 2)   This demonstrates clearly that after injection, the finding of a few bacilli in slightly granulomatous areas in the organs of the rat, does not prove that the organism injected is the organism of rat leprosy   Similar results were reported by Frazer (1912, 1913 and 1914) and, as he pointed out, a misinterpretation of these findings is probably responsible for most of the reports of experimental transmission of human leprosy to animals   The injection of the organisms of rat leprosy in reasonable quantity into a rat is followed in time in every case by the progressive development of generalized lesions, teeming with acid-fast bacilli when fully developed, and ultimately causing death

This infection can be transmitted to rats in series for an indefinite period. This is the one and only criterion for judging whether any bacillus is that of rat leprosy. Our own strain of rat leprosy was obtained from one rat eight years ago, and since then hundreds of rats have been infected in series.

The cultivation of M leprae muris

Though many workers have attempted this, only a few have claimed success. Dean (1905) grew an acid-fast diphtheroid from rat-leprosy material on one occasion only. Marchoux and Sorel (1913) reported marked multiplication of bacilli in pieces of excised leprous gland embedded in culture media but failed to produce any subcultures. The 'gland cultures' showed filamentous forms and branching forms. Marchoux and Sorel succeeded in getting some growth on the spleen of a rat sterilized by heating to 110°C and partially digested by trypsin, by inoculating it with material from a 'gland culture'. The growth appeared within a few days and then ceased suddenly, and at the end of six weeks the bacilli were reported as granular and dead.

Hollmann (1912) obtained a culture by Clegg's method but Currie and Hollmann (1913) reported failure to cultivate the organism of rat leprosy by ordinary methods and also by the method of Clegg. They reported that as the result of many months' work they had not secured a single culture of an organism which they considered to be that of rat leprosy. In one attempt they grew an acid-fast streptothrix from an ulcerated lesion but considered it a contamination, as is very likely in such a lesion.

Bayon (1914) reported, 'From the spleen of a rat injected with ground up lesions of a spontaneously affected rat, a white moist creamy growth was isolated which under the microscope showed itself to be a pleomorphic absolutely acid-fast rod indistinguishable from the culture obtained from a leper. This culture on injection into rats produced the identical visceral lesions which I have observed in rats injected with the virus from spontaneously infected animals; these lesions are produced again by an enormous quantity of acid-fast rods with little or no tissue reaction'. The medium used was fish-juice-agar. Growth occurred in 14 days.

Walker and Sweeney (1929) reported finding acid-fast bacilli in 37 of 74 rats examined and showing no gross lesions of rat leprosy. They obtained cultures from 24 of these 37 rats on Musgrave and Clegg's medium incubated at 22°C to 25°C. The organisms cultivated were pleomorphic 'like those of human leprosy'. They hold that the two organisms are identical, that human and rat leprosy are the same disease and that both are due to a soil actinomyces. No report is made of animal inoculations with the culture. The findings of Bayon and of Walker and Sweeney have not been verified by any other workers as far as we know. Most other workers report absolute failure to cultivate the organism; in this laboratory attempts to obtain cultures by the method of Walker and Sweeney have given absolutely negative results.

Uchida (1923) isolated from rats five strains of acid-fast organisms. Thanks to the kindness of Dr M Nagayo, Director of the Government Institute for Infectious Diseases, Tokyo, we have secured subcultures of these five strains. Unfortunately

one was contaminated and one was dead on receipt   The other three have been easily subcultured, growth being visible in twenty-four hours on Petragnini's and Hohn's media   We have injected emulsions of these bacilli into rats, but have obtained no evidence of the progressive systemic infection which is characteristic of experimentally-induced rat leprosy

Ota and Asami (1932) considered that Uchida's cultures were true cultures of rat leprosy   They have isolated on Petragnini's and Petroff's media, acid-fast bacilli from eleven rats   Colonies appeared after periods varying from 5 to 110 days   The bacilli were acid-fast in the original culture but in subcultures became less and less acid-fast   All the cultures when injected into rats were reported as producing the same changes as are seen in naturally-infected rats   The glands become enlarged and sections show masses of acid-fast bacilli inside macrophage cells   *No report was made of infection of rats in series, nor of the development of generalized progressive disease*

Cilento and North (1931) reported that in one of many attempts, one primary culture on Dorset's egg medium was obtained   The isolated bacilli injected into young rats were reported to have produced typical lesions   We have secured from Dr Cilento some tubes of his culture   It showed an acid-fast cocco-bacillus, easily subcultured, but when injected into rats producing no generalized systemic disease but merely the lesions described above as typically produced by any non-pathogenic acid-fast organism living or dead

We think that many of the reports of culture of *M leprae muris* are due to the fact that non-pathogenic acid-fast bacilli are sometimes found in rats with no visible lesions of rat leprosy   It is noticeable that some workers (e g , Walker and Sweeney) have reported isolation and cultivation of *M leprae muris* from material of this kind   Such bacilli may be cultured and when injected into animals may persist and produce a certain degree of granulomatous change in the tissues   This does not prove that the organism isolated is that of rat leprosy   The criterion is the development in rats in series, of a systemic infection with marked lesions crammed with acid-fast bacilli, the disease being transmissible indefinitely to rats in series It is noticeable that in none of the supposedly successful attempts at culture has this criterion been used to prove the genuineness of the culture

In this Department we have made many attempts at culture of *M leprae muris*, from rats with marked lesions of rat leprosy, using the common media, using those media (Hohn's, Petragnini's, Lowenstein's) which have been reported in recent years as being the best for acid-fast organisms, and using gas concentration methods as advocated by Soule and McKinley (1932)   On not a single occasion has an acid-fast organism been isolated, all the tubes, except those contaminated, showing no true growth at all

We have on many occasions produced the appearance of culture   Acid-fast bacilli have extraordinary powers of persistence on media, and when, as is usual, rich emulsions of bacilli are used for seeding the media, acid-fast bacilli can be detected on the surface of the media for as long as six months, and these bacilli if not dry are capable of infecting rats   Also, when emulsions of tissue containing bacilli are used for seeding media, the tissue cells inoculated undergo lysis, and swell up, giving the appearance of a colony, in which large n   of acid-fast bacilli may be found   Plate XII, fig 3, is a photograph of t   es

inoculated one month previously with a large drop of an emulsion made from the spleen of an infected rat   In these two tubes there is no contamination, but merely swollen and lysed splenic cells and the acid-fast bacilli which were seeded on the media   Subculture gives negative results

Freidheim (1929) reported attempts at culture of *M lepræ muris* in tissue culture with negative results

We believe that *M lepræ muris* has not yet been cultivated *in vitro*   Tissue-culture methods seem to be the most likely methods to succeed   We propose to follow this method of work

A filterable form of M lepræ muris

Markianos (1929) was the first to report investigations on the matter, following the reports by many workers of tubercle being transmitted by the filtrate of bacillary emulsions filtered through Chamberland filters

He used Chamberland L2 filters, for filtering an emulsion of bacilli obtained by grinding up leprous tissue from a rat   Filtration was done under a pressure of 25 to 30 centimetres of mercury   His findings were as follows —

Injected into rats the filtrate caused inflammation in the local lymphatic glands, and later, lesions in the viscera   The filtrable virus first develops into acid fast granules, later into granular bacilli and later still into true bacillary forms   The bacillary form may be found in twenty days in young rats and in two or three months in adult rats   The granular forms commonly seen in rat leprosy are, Markianos considers, a stage between the filtrable virus and the true bacilli   He quotes Marchoux as supporting this opinion

We have attempted to verify the findings of Markianos   In work of this kind there are many fallacies to be avoided   One source of error is the faulty candle , a second is accidental infection of experimental rats in an animal room in which there may be dozens of rats in the advanced stages of rat leprosy , a third is that a few non-pathogenic acid-fast bacilli may sometimes be found in the glands of healthy rats   We have taken every precaution against these sources of error, but we consider that in work of this kind, little notice should be taken of a few slightly positive results, that at least 50 per cent of positive results are necessary for proof of the hypothesis of the filtrable form, and that a positive result should only be recorded if there follows a progressive systemic infection with lesions showing masses of acid-fast bacilli   The filtration was done by Captain Pasricha, I M S   The candles were very carefully tested before use, and in one experiment the material was filtered through two different candles in series in order to minimize the possibility of error due to a faulty candle   Candles L2 and L3 were used, and also L5 as a control   The size of the pores of candle L2 is said to be sufficient to keep back large bacilli but not the smaller bacilli   In all 27 rats were injected with material filtered through L2 candles, 26 rats with material filtered through L3 candles and 12 rats injected with material filtered through L5 candles   Of the L2 series 2 rats showed a few acid-fast bacilli and 0 showed a general systemic infection   Of the L3 series 2 rats showed a few acid-fast bacilli and 1 showed a general infection   Of the L5 series 0 showed acid-fast bacilli and 0 showed a general infection   We consider these results are against there being a filtrable form of *M lepræ muris*   The positive results are probably explained by experimental errors

The viability of M lepræ muris

Various workers [Muir and Henderson (1927), Marchoux and Sorel (1918), Marchoux and Chorine (1932)] have reported on the viability *in vitro* of *M lepræ muris* under various conditions　We have nothing to add to their findings except in two matters　Firstly, we have found that *M lepræ muris* as long as it is kept moist remains viable and pathogenic to rats for several months outside the body

Secondly, we have investigated with some care the action of the sodium salts of the fatty acids of hydnocarpus oil on *M lepræ muris*　This is a matter of considerable importance, and will be dealt with separately

The action of the sodium salts of the fatty acids of hydnocarpus oil on M lepræ muris

The use of chaulmoogra and hydnocarpus oil in the treatment of human leprosy was at first purely empirical　Walker and Sweeney (1920) tried to supply a scientific basis for this form of treatment

They investigated the action of derivatives of chaulmoogra oil on acid-fast bacilli in cultures

They found that sodium hydnocarpate in a dilution of 1 in 75,000 killed *M lepræ muris* in twenty-four hours　They considered that these and other similar findings supplied 'a scientific basis for the use of chaulmoogra oil and its products in leprosy'　These results appear very definite and conclusive, and they have received wide recognition　An examination of their experimental data shows certain grounds for criticism　Their crucial experiments were performed with a culture called '*B lepræ muris (Hollmann)*' *　Secondly, the criterion of the bactericidal effect used by Walker and Sweeney was the failure of treated bacilli to grow in culture and subculture　This is very unsatisfactory　Some acid-fast bacilli need special conditions for growth, and the mere failure to grow does not prove that they have been killed　The real criterion of death is the failure on injection into susceptible animals to produce the disease　Walker and Sweeney attempted to demonstrate this with *M tuberculosis* but their work was quite inconclusive　These are the weak points of Walker and Sweeney's work　We have carried out experiments to remedy these defects

In our experiments we have used two different preparations of hydnocarpus oil (which is identical with chaulmoogra oil)　(1) the sodium salts of the total fatty acids of hydnocarpus oil, (2) Alepol, a proprietary preparation which consists of the sodium salts of a selected fraction of the lower melting point fatty acids of hydnocarpus oil

A rat in the advanced stages of rat leprosy was sacrificed, and an emulsion made from the spleen　This emulsion was rich in acid-fast bacilli　Different lots of this emulsion were then used with dilutions in normal saline of sodium hydnocarpate and Alepol in various strengths and for varying periods　The mixtures

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* This organism was isolated in 1912 (Hollmann, 1912) but in 1913 Currie and Hollmann wrote of their attempts to culture *M lepræ muris* ' our results have been entirely negative ' and do not mention the claim made by Hollmann in the previous year　It appears that Hollmann changed his opinion about the genuineness of his culture

were then centrifugalized, and washed repeatedly to remove the sodium hydnocarpate, and the treated and washed bacilli were then injected into healthy rats, which were kept for several months, some as long as one year. Rats dying naturally or sacrificed were carefully examined post-mortem, smears being made from the various organs, and tissues being sectioned and stained for acid-fast bacilli. The finding of merely a few acid-fast bacilli was not taken as indicating the presence of rat leprosy. The presence of a generalized leprous infection was the standard adopted for proving that the bacilli injected were viable and pathogenic.

The results of two such experiments may be summarized as follows :—

Bacilli were with treated dilutions of sodium hydnocarpate varying from 1 in 20 to 1 in 2,000,000 for periods varying from 3 hours to 20 hours, and subsequently injected into rats

Eighty four rats were used and every rat which lived for more than two months showed a generalized and progressive infection, though in some rats the development of the disease appeared to be retarded. Similar results were obtained with bacilli treated with sodium oleate, which suggests that the only direct action is a simple soap action

These experiments show that even in high concentration acting for long periods, the bactericidal action of sodium hydnocarpate on *M. lepræ muris in vitro* is extremely slight or absent. This salt may have the power of inhibiting growth of *M. lepræ* during the time of its action ; regarding this we can say nothing ; but in concentrations far higher than can possibly be obtained in the tissue fluids of the human or animal body, it does not kill the bacilli *in vitro* in 20 hours

The fact that sodium hydnocarpate does not kill *M. lepræ muris in vitro* does not prove that it has no action *in vivo*. Quinine does not kill malarial parasites *in vitro* , antimony does not kill *Leishmania in vitro* , but they both have a marked action *in vivo*. Nevertheless, a similar bactericidal action of sodium hydnocarpate *in vivo* we consider to be extremely hypothetical

CLINICAL AND PATHOLOGICAL

Naturally-acquired rat leprosy

This has been very well described by Stefansky (1903), Dean (1905) and Marchoux and Sorel (1913). Two forms are seen in nature (1) the 'glandular' form with no lesions in the skin and internal organs, but marked enlargement of the glands of the axilla and groin, the glands containing enormous numbers of acid-fast bacilli, (2) the musculo-cutaneous form which is probably a more advanced stage of the glandular form and is characterized by marked lesions of the skin, subcutaneous tissue and of the underlying muscles ; visceral lesions are reported as rare

Experimentally-induced rat leprosy

Rat leprosy is usually induced by the injection of emulsified leprotic tissue from an infected rat. It can also be produced by merely keeping a rat in the same cage with a heavily infected rat (Currie and Hollmann, 1913), by scarification of the epidermis and touching the area with leprous material, by smearing leprous material on freshly depilated skin (Marchoux and Sorel, 1913) and sometimes by feeding rats on rat leprosy material. Injections may be given subcutaneously, intraperitoneally, intravenously or intracardially. The lesions, and the time taken

to produce them, vary with the method of injection used and the dose of bacilli given

If the injection is given into the subcutaneous tissue, lesions develop first at the site of inoculation, then in the lymphatic glands, and later in the internal organs, and in advanced cases practically all the tissues of the body may be affected   With subcutaneous injections the time taken for the full development of the disease is usually at least a year even when as much as 1 c c of rich emulsion containing many millions of bacilli is injected, and with smaller doses an even longer period is necessary, and many rats die from other causes while the disease is still in a mild form

Intraperitoneal injection produces lesions in the omentum, in the lymphatic glands, in the abdomen, and later in the liver and spleen, as well as more generally   The injection of 1 c c of rich emulsion produces marked lesions in a few weeks, in three months the infection is usually general, and in about nine months most rats have died either directly or indirectly as the result of rat leprosy

Even quicker results are obtained by intravenous or intracardiac injections, as the infection from the start is general and, even with small doses of bacilli, marked general lesions are rapidly produced and most animals are dead within six months

Whatever method of injection is used, the typical ' glandular ' and ' musculo-cutaneous ' forms described in natural rat leprosy are not reproduced   However, a form resembling the natural ' glandular ' form we have produced in experimental rats by intradermal injection of very small quantities of emulsion   By this method, at the end of about a year, there is produced a marked general glandular enlargement, but in addition there is often a lesion at the site of injection, which is not seen in the natural ' glandular ' form   We have not been able to produce experimentally a form resembling the natural ' musculo-cutaneous ' form with multiple lesions in the subcutaneous tissue , it is possible that multiple small injections into the skin might produce it

In experimental rat leprosy we have found that many tissues which are apparently normal, show numerous bacilli when smears are made, and very definite microscopic lesions on sectioning   We suspect that the same thing is true in natural rat leprosy, and that more careful examination would show visceral lesions to be more common than has been reported

Pathology of experimental rat leprosy

Skin and subcutaneous tissues —The lesions in the subcutaneous tissue and the skin are essentially the same as those described in natural rat leprosy and need not be described here

Regarding the distribution of lesions in the skin, we have found that the visible lesions in experimental leprosy are always confined to the skin near the site of inoculation   There is no general invasion of the skin until the animal is moribund with a massive general infection, and even then the multiple skin lesions are merely post-mortem findings detectable by smears or sections but not clinically   These microscopic lesions in the skin take the form of small areas of leproma round the blood vessels in the skin, apparently produced by emboli of bacilli being deposited

in the capillaries of the cutis   We have found these lesions in the skin in nearly every part of the body

The lymphatic glands —The lesions of the glands are essentially the same as those described in natural rat leprosy, except that the very marked glandular enlargement is rarely seen   As already noted, however, we have been able to produce this marked general enlargement by injection of very small doses of bacilli into the skin of the abdomen

The muscles —We have never seen any actual infection of the muscle fibres in experimental rat leprosy   Lesions are found affecting muscles near the site of injection, but the granuloma always arises in the connective tissue outside and between the muscle fibres, and only affects the muscles indirectly by pressure We have seen such lesions in the muscles underlying the skin at the site of injection, in the abdominal muscles infected by leakage in intraperitoneal injections, and in the pericardium and heart muscle infected similarly in intracardial injections (*see* Plate XII, fig 4)   These findings agree with those of Marchoux and Sorel (1913) and disagree with those of Stefansky (1903) and Dean (1905), who reported multiplication of bacilli inside muscle fibres   This appearance we believe to be an artefact

Internal organs, etc —The great interest of experimental rat leprosy lies in a study of the internal lesions   Practically all the internal organs and tissues are affected in advanced general rat leprosy, but the first tissues to show marked change, and the tissues which are most affected all through, are the liver, spleen and bone marrow   We base this finding on the results of observation of rats after intra-cardiac injection, in which case the bacilli are at once liberated into the blood stream   Bacilli are found in these organs within a few minutes, definite but slight pathological lesions are detectable within a few days, marked lesions within a few weeks   The liver, the spleen and the bone marrow are those tissues in which the cells of the reticulo-endothelial system are most numerous, and as will be described later, the essential change in these lesions is infection and proliferation of these particular cells   We believe that rat leprosy is essentially an infection of the reticulo-endothelial system of the rat

The liver —This organ is perhaps the easiest site in which to study the nature of the pathological changes produced by leprosy   If a series of rats is given an intracardiac injection of $\frac{1}{4}$ c c of a rich rat-leprosy emulsion and one rat is sacrificed 15 minutes and others at 12 hours, 24 hours, 4 days, 1 week, 2 weeks, 1 month, 2 months, etc , after injection, and if sections are cut of the tissues, particularly of the liver, a very clear demonstration is obtained of the process of invasion of this organ by the disease

In the animal killed a few minutes after injection some bacilli will be found after careful search in sections of the liver   They will be found singly or in pairs and already even in fifteen minutes they will have been ingested by the Kupffer cells lining the sinuses of the liver

In a few days proliferation of the Kupffer cells will be seen, though the bacilli are still few in number   In two or three weeks, the small masses of proliferated Kupffer cells are easily detectable with the low power of the microscope   This proliferation of cells continues, and the bacilli also proliferate, and after a few more weeks is seen the characteristic appearance of definite masses of greyish colour, contrasting with the brown of the liver tissue, and easily visible to the naked eye

The liver becomes enlarged, the diseased masses increase in size and by the time of death the liver is very big and full of granulomatous and caseous masses The liver tissue is affected only secondarily by the pressure of the granulomatous masses (*see* Plate XII, fig 5, and Plate XIII, figs 6 and 7)

The spleen —In the spleen similar changes are seen The reticulum cells of the spleen ingest some of the bacilli a few minutes after intracardiac injection Within a few days, proliferation of the cells is detectable microscopically and after three or four weeks marked multiplication of the bacilli is seen At this stage the spleen appears normal to the naked eye It is noticeable that in sections the changes are not seen in the Malpighian corpuscles but in the reticulum in between the corpuscles, indicating that lymphoid tissue is unaffected but the reticulum cells are Later, the small areas of granulomatous cells full of bacilli coalesce forming definite nodes visible first microscopically and later macroscopically The spleen becomes large and is studded throughout by these round white nodes (*see* Plate XIII, fig 8)

The bone marrow —The bone marrow also frequently shows infection within a few minutes of injection, bacilli being found inside the reticulum cells As in the spleen and liver this infection steadily increases until within a few weeks the bacilli are very numerous and in three or four months the bone marrow consists almost entirely of cells crammed with acid-fast bacilli

The other tissues, which we have found infected after intracardiac injection, are the lymph glands, the lungs, the pericardium, the testes, the ovaries, the seminal vesicles, the uterus, the lungs and the skin The tissues never affected are the peripheral nerves, and the muscles (affected only secondarily) The kidney usually shows no lesions

In our experience (contrary to that of Currie and Hollmann) infection of the lungs is a late manifestation of leprosy Even intracardiac injections if given in the left heart produce no early lesions of the lung , marked lung affection usually occurs only late in the disease It is quite true that when bacilli are circulating in the blood, some frequently lodge in the lungs, but they seem to be carried thence to the mediastinal glands which are frequently markedly involved, and progressive lung lesions are not usually produced till late Sometimes experimental rats die of pneumonia, and consolidation of the lungs is found and smears from the lungs show some acid-fast bacilli Section of the lung in these cases usually shows an infection of the lungs by other organisms, and only a few acid-fast bacilli in the consolidated tissue in the lungs The leprosy infection is not the cause of the lung condition but some acid-fast bacilli have lodged in the lung tissue otherwise diseased We believe that this is the cause of the anomalous findings reported by Currie and Hollmann In really leprous lesions of the lung, acid-fast bacilli are found in enormous numbers and not in the small numbers reported by Currie and Hollmann

We need not describe in detail the lesions of the ovaries, testes, seminal vesicles and uterus , massive infection of the interstitial tissue in these sites is frequently seen in advanced experimental rat leprosy

We have already referred to the lesions at the site of injection , one form of this is the leprotic pericarditis seen after intracardiac injection The heart muscle is not affected except secondarily by the extension of the granuloma from the pericardium in between the heart muscle fibres Another form is the tremendous

PLATE XI

Fig 2 Section of liver of rat injected one year previously with massive doses of *M leprœ muris* killed by heat in an autoclave Note 'giant cell' full of bacilli

Magnification 600   Stain   Ziehl Neelsen modified

Fig 1   Section of mediastinal gland of a rat injected six months previously with autoclaved human leprosy bacilli   Note granuloma and bacilli in gland There is no systemic disease

Magnification 400   Stain   Ziehl Neelsen modified

PLATE XII

Fig 3   Pseudo culture of *M lepræ muris*

Fig 5   Section of liver of rat a few days after
intracardiac injection of rat leprosy bacilli   The bacilli
are seen inside Kupffer cells

Fig 4   Section of pericardium and heart muscle of rat
several months after intracardiac injection of *M lepræ
muris*   The granular looking mass is the enormously
thickened pericardium crammed with acid fast bacilli

PLATE XIII
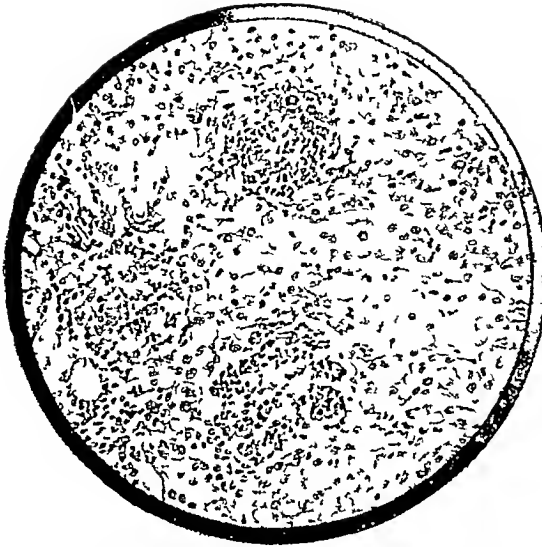
Fig 6   Section of liver of rat three weeks after intracardiac injection of *M lepræ muris*   Note marked granulomata   (The bacilli are unstained)

Magnification   600   Stain, Hæmatoxylin and eosin
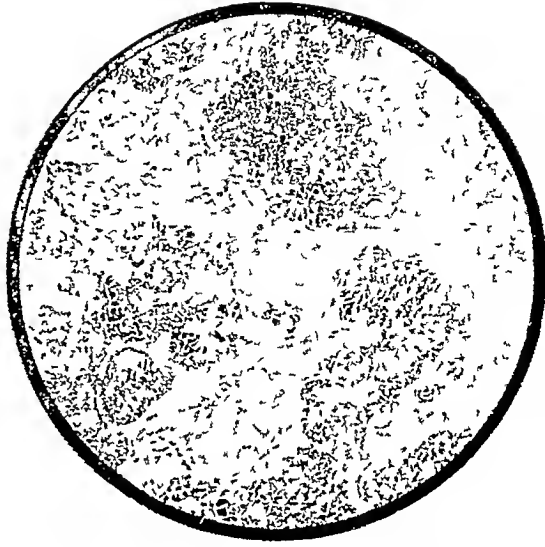
Fig 7   Section of liver with marked leprous lesions   The dark masses consist of cells crammed with acid fast bacilli

Magnification   Low power   Stain   Modified Ziehl Neelsen

Fig 8   Spleen enormously enlarged and studded with lepromata which consist of cells crammed with acid fast bacilli

invasion of the omentum seen after intraperitoneal injection   The omentum after
a few months becomes an enormous thick mass consisting almost entirely of proli-
ferated reticulum cells crammed with bacilli   This makes intraperitoneal injection
a most useful method for obtaining material rich in bacilli, for an emulsion made
from such an omentum contains enormous numbers of bacilli and few cells   We
have already referred to the diffuse infection of the skin found post-mortem and not
clinically, and occurring only in very late stages of rat-leprosy infection

The cytology of rat leprosy

In rat leprosy the bacilli are almost entirely intracellular   The bacilli are
contained inside large round or oval cells which have a large vesicular nucleus
staining rather faintly, and abundant protoplasm often showing an apparent vacuo-
lation   The question arises as to what is the origin and nature of these cells   We
have already stated reasons for believing that these cells are of reticulo-endothelial
origin   In the skin they apparently originate from the connective tissue histio-
cytes , in the glands from the reticulum histiocytes, in the liver from the Kupffer
cells, in the spleen from the reticulum cells, and in the bone marrow from the reti-
culum cells, and similarly in other organs and tissues   We have tried to demon-
strate this clearly by vital staining methods, injecting an infected animal with
trypan blue   This was done first by Oliver (1926) in studying the skin lesions
of rat leprosy, and she found that the so-called ' lepra ' cells were the cells which
took up the trypan blue dye, thus indicating that they are essentially histiocytes
She found, however, that the cells full of bacilli took up little of the dye, those with
a few bacilli took up more and those with no bacilli took up most   Her findings
were verified by Henderson (1928)   We have injected trypan blue into rats in the
comparatively early stage of a general rat-leprosy infection produced by giving an
intracardiac injection of bacilli   Here the picture is much more complete and
convincing   In all the tissues, the cells which ingest the dye are precisely the cells
which also ingest the bacilli, the dye is most abundant in those tissues in which
the bacilli are most abundant, and there is a complete correspondence between
the dye and the bacilli

The question then arises as to what happens to the ingested bacilli   Are they
killed or rendered inactive ?   From a very careful study of the disease we have
definitely concluded that the bacilli inside the cells are not destroyed or rendered
inactive , on the contrary the cells seem to provide just the conditions necessary
for the multiplication of the bacilli   The cell ingests the bacilli but cannot digest
them , the bacilli multiply, the cell swells up, the bacilli multiply still further and
eventually the cell is enormously distended by the hundreds of bacilli inside it
The cell envelope may burst and thus liberate bacilli which are promptly ingested
by other similar cells, and this process is repeated   While this is proceeding the
neighbouring cells multiply, and possibly there is an infiltration of wandering
histiocytes also, but the progress of multiplication of bacilli and infection of fresh
cells continues   Finally, the reticulo-endothelial system is exhausted, the defence
mechanism breaks down, and there is rapid spread of infection throughout the
body, followed by death

Multi-nucleated giant cell formation we have frequently observed   These
cells obviously arise from the reticulum cells   It may be that several cells coalesce

to form the giant cell, or in a single cell division of the nucleus occurs without division of the cytoplasm   (It is noticeable that the multi-nucleated giant cell of rat leprosy always contains large numbers of bacilli, while the multi-nucleated giant cell of human leprosy in our experience very rarely contains bacilli)

SUMMARY

1   A comparison is made between human and rat leprosy

2   Various reports of successful culture of the organism are critically reviewed Failure to reproduce the disease by injection of various so-called cultures is reported Many unsuccessful attempts at culture are reported   The conclusion is drawn that the bacillus has not yet been cultured

3   Attempts to detect a filtrable form of the organism have met with no success

4   Sodium hydnocarpate is found to have little or no bactericidal effect on the bacillus *in vitro* even in dilutions of 1 in 20 acting for 20 hours

5   The pathology of experimentally-induced rat leprosy is discussed fully Rat leprosy is considered to be essentially a disease of the reticulo-endothelial system

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FOUNDATION OF A BIOCHEMICAL METHOD OF STANDARDIZING VITAMIN D OR IRRADIATED ERGOSTEROL

BY

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Up to the present the standardization of vitamin D or irradiated ergosterol has been based upon the calculation of the minimum amount of the substance that is required to cure rickets artificially produced in rats, and for this either the animals had to be killed or the help of x-rays was an absolute necessity Of these two methods, diagnosis by means of x-rays is without doubt the better and has, therefore, been accepted as the standard

But, as the handling of x-ray apparatus requires knowledge and a high technical skill and as the cost of the apparatus is so high that every laboratory cannot afford to install one, we have tried to find some simple biochemical method of standardization of vitamin D or irradiated ergosterol

This biochemical method is based upon the fact that when rickets is artificially produced in rats or in any other lower animal, both the calcium and phosphorous contents of the blood gradually fall and when signs of active rickets appear in the bones, both calcium and phosphorous contents reach a certain definite minimum figure, so that the product of these two figures is very nearly constant At this stage, with proper control, different doses of vitamin D are administered to different groups of animals, and the minimum amount of substance that raises the product of the figures of calcium and phosphorus to the original level within a definite period of time, is fixed as the standard

Though rats have been found to be suitable for standardization of vitamin D by the x-ray method, rabbits have been found to be still more suitable for this biochemical method   The reason for this is that sufficient blood for estimation can be very easily drawn from these animals by heart-puncture, which is not always the case with rats   In practice young rabbits of two months old were selected

The greatest objection that can be raised against using rabbits for such experiments is that these animals show marked variation in their calcium and phosphorous contents when estimated in the morning and in the evening   It has been found, however, that the estimation of calcium and phosphorous contents in fasting animals shows practically no diurnal variation   So, in the present series of experiments, animals were allowed to fast for 12 hours before the blood was drawn, which was done in the morning between 9 and 9-30

200   *Biochemical Method of Standardizing Vitamin D*

Befoie the experimental diet was given to the animals, the calcium and phosphorous contents of the blood were estimated   On an average the calcium content was found to be 12 mg and the phosphorus 5 mg , so that the product of these two figures was appioximately 60 mg

The animals were then shut off fiom light and were fed with Steenbock's rachitogenic diet consisting of ground yellow maize 76 per cent, wheat gluten 20 per cent, calcium caibonate 3 per cent and sodium chloiide 1 pei cent   After two weeks the animals weie examined undci x-rays and signs of early rickets were manifest At this stage the calcium and phosphoious contents of the blood were estimated and on an average calcium was found to be 10 mg and phosphorus 4 mg , so that their product was approximately 40 mg

The experiment was continued for another week, and the animals were again examined under x-rays   Signs of active rickets were fully evident, and on estimation calcium and phosphorous contents were found to be 9 mg and 3 mg respectively, 1 c , then product was 27 mg

From this point the animals were given different doses of vitamin D and after 7 days the calcium and phosphorous contents of the blood were estimated   The animals were examined under x-rays for signs of healing rickets   Between 7 and 10 days early signs of healing rickets appeared in the bone and estimation of the calcium and phosphorus also showed increased figures of 10 mg and 4 mg respectively, so that their product was 40 mg

The animals were completely cured of rickets aftei a period of 15 to 18 days, as diagnosed by x-ray examination, and estimation of the calcium and phosphorus also showed normal figures of 12 mg and 5 mg respectively, so that their product was 60 mg

The smallest amount of substance that could raise the product of calcium and phosphorus of the blood from 27 mg to 40 mg within 7 to 10 days, oi raise the figure to 60 mg within 15 to 18 days, was taken as the standard

TABLE I

Calcium and phosphoious contents of the blood normally present in young rabbits of 2 months old

Number	Calcium in mg	Phosphorus in mg	Calcium × phosphorus	X ray examination
1	12 2	4 8	58 6	Bone normal
2	12 0	5 2	62 4	Do
3	11 8	5 2	61 4	Do
4	11 8	5 0	59 0	Do
5	12 4	5 0	62 0	Do
6	11 6	5 4	62 6	Do
7	11 8	5 2	61 4	Do
8	11 4	5 0	57 0	Do

TABLE II

Calcium and phosphorous contents of the blood after 2 weeks of Steenbock's rachitogenic diet

Number	Calcium in mg	Phosphorus in mg	Calcium × phosphorus	X ray examination
1	10 2	4 0	40 8	Early signs of rickets present
2	10 2	4 2	42 8	Do
3	10 0	4 0	40 0	Do
4	9 8	4 2	41 2	Do
5	10 0	4 0	40 0	Do
6	9 6	4 2	40 3	Do
7	10 0	4 0	40 0	Do
8	9 8	4 0	39 2	Do

TABLE III

Calcium and phosphorous contents of the blood after 3 weeks of Steenbock's rachitogenic diet

Number	Calcium in mg	Phosphorus in mg	Calcium × phosphorus	X ray examination
1	8 0	3 0	27 0	Active signs of rickets present
2	8 8	3 0	26 4	Do
3	8 8	2 8	24 6	Do
4	9 0	2 8	25 2	Do
5	9 2	3 0	27 6	Do
6	9 0	3 0	27 0	Do
7	8 8	3 2	28 2	Do
8	8 8	3 0	26 4	Do

TABLE IV

Calcium and phosphorous contents of the blood after 7 days of administration of irradiated ergosterol

Number	Irradiated ergosterol per kg per day, in mg	Calcium in mg	Phosphorus in mg	Calcium × phosphorus	X ray examination
1	0 01	9 2	3 4	31 3	No healing of rickets
2	0 01	9 0	3 4	30 6	Do
3	0 02	9 6	3 6	34 6	Do
4	0 02	9 4	3 6	33 0	Do
5	0 03	9 8	3 8	37 2	Signs of healing present
6	0 03	9 6	4 0	38 4	Do
7	0 04	9 8	4 2	41 2	Do
8	0 04	10 0	4 0	40 0	Do

TABLE V

Calcium and phosphorous contents of the blood after 15 days of administration of irradiated ergosterol

Number	Irradiated ergosterol per kg per day, in mg	Calcium in mg	Phosphorus in mg	Calcium × phosphorus	X-ray examination
1	0 01	10 4	4 8	50 0	Healing not complete
2	0 01	10 8	4 6	49 9	Do
3	0 02	11 4	4 8	54 7	Do
4	0 02	11 6	4 8	55 9	Do
5	0 03	11 8	5 0	59 0	Healing complete
6	0 03	12 0	4 8	57 6	Do
7	0 04	12 0	5 0	60 0	Do
8	0 04	11 8	5 0	59 0	Do

A SIMPLE METHOD OF GROWING HOOKWORM LARVÆ.

BY

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INTRODUCTION

THE culturing of hookworm larvæ is an important matter in the investigation of many aspects of the hookworm problem, and numerous conclusions have been based on information obtained from the results of a variety of methods of culturing these larvæ   Most workers have followed Looss' original method to the extent that they have employed Petri dishes in which to grow their cultures, although they have possibly modified the culture medium and his method of extracting larvæ Lane (1928) made the important observation that larvæ are prone to migrate from cultures made in these dishes, but there seems to be no rule whereby the actual number that may be expected to migrate can be estimated, as the numbers found in his traps varied widely without apparent reason   One possible explanation of this variation is that the larvæ of *Ancylostoma duodenale* have different migratory habits from those of *Necator americanus*, and there is no information on the proportions of these two species of worms in the patients from which Lane obtained the stools that he used   Whatever the explanation may be, the fact of the occurrence of migration is of great importance as it invalidates our earlier conclusions, based on the number of larvæ recovered from Petri dish cultures

Although the Petri dish has been almost universally used for making hookworm cultures in the laboratory, a little thought will make one realize that in them the larvæ are being grown under highly unnatural conditions   They have to live and grow in a layer of earth or charcoal not more than 1 cm in depth which is practically all the time saturated with water, because any water added can only evaporate as there is no substratum into which excess moisture can drain away   It has been argued that because hookworm larvæ to remain alive must always be covered with a film of water, it does not matter how much water the soil they are living in contains Hookworm larvæ, however, are not truly aquatic animals and Maplestone (1926) showed conclusively that excess of water in the soil was unfavourable for their development   This observation, which was made in West Africa where the soil

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used was a highly porous and readily-drained laterite, has since been confirmed in Bengal where the soil is heavy, non-porous and consequently drains very badly Another unnatural condition to which these cultures must of necessity be subjected in a cool climate is that they have to be kept in an incubator and are thus continually under an unvarying temperature, night and day, throughout the whole period of their growth, but unfortunately this cannot be avoided when working in a temperate climate   It appeared possible that the highly unnatural conditions to which these larvæ had been subjected might be the cause of their migration in an attempt to find more congenial surroundings

Lane made his original observations with Petri dishes standing in larger ones containing water, and it was in these that the larvæ were trapped   Later in the same year (Lane, 1928a) he demonstrated a special vessel he had designed for culturing hookworm larvæ and which he considered to be essential for the estimation of the length of life of hookworm larvæ in soil   His apparatus consists of a jar, the open end of which is surrounded by a fixed moat in which water can be kept to entrap any larvæ that leave the culture   No dimensions are given but the size of the drawings suggests the probability that it is reproduced in its natural size

In a full discussion of the question of culturing hookworm larvæ Lane (1932) refers to 'the inescapable need to trap cultures'   This statement is of course correct if cultures are put up in Petri dishes, but it does not seem to have occurred to this worker that the employment of a different technique in culturing might do away with this necessity   Maplestone (1926) briefly described the technique he devised for studying certain aspects of the development of hookworm larvæ, but his work has apparently been overlooked as no mention of it appears in any of Lane's papers on the subject   It was devised with the object of providing a more natural environment for the hookworm larvæ to live in, than the usual badly-drained Petri dish cultures   At the time no special stress was laid on the method because the writer, like all other workers on the subject, was then unaware that larvæ migrated from cultures   Since the publication of Lane's work this method has been tested to find if hookworm larvæ migrate from cultures of the type to be described, and as it has been found that if proper precautions are taken larvæ do not migrate from cultures of this type the method has been elaborated and improved   As the method is simple, the materials needed can be obtained almost anywhere and the apparatus can be easily made, it is proposed to give a detailed description and some of the results obtained by its use

METHOD OF CONSTRUCTING THE APPARATUS

A quantity of fine gauze made of non-corrosive wire, such as brass, copper or bronze, and with a mesh of about 1 mm square is required

Square shallow baskets are made out of this gauze in the following manner They can of course be made of any size desired depending on the size of the cultures to be made, and the dimensions given below are those that for general purposes we have found a very convenient size and one that will easily accommodate up to 5 c c of stool —

A square piece of gauze 12 cm by 12 cm is cut out and in its centre a square 6 cm by 6 cm is marked with a pencil   Diagonal cuts are made from the corners

of the gauze square to the corresponding corners of the square marked in its centre (Text-figure)   The gauze is quite easily cut with an old pair of scissors   Using the central square as the base, the sides are bent up at right angles to it   The result of this is that at each corner there are two triangular portions of gauze projecting one beyond the other   These are bent around each other   forming right-angled corners, and they are tied to the sides by one or two twists of wire unravelled from the stock piece of gauze, and the small basket is complete   A valuable addition is a handle made from a length of wire formed by two or three strands unravelled from the stock piece and twisted together   Such a loop renders handling of the cultures easy and rapid without any danger of the hands becoming contaminated with the earth in the culture or, more important, with infective larvæ

TEXT FIGURE
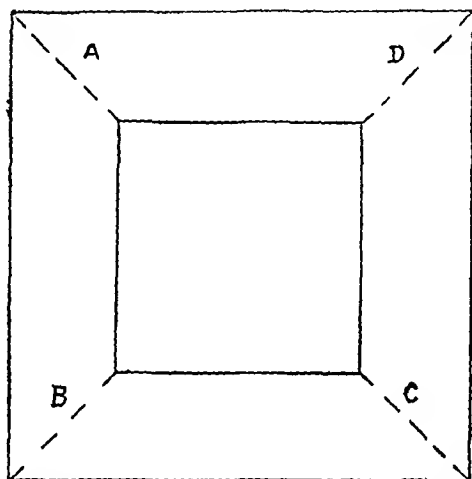
Diagram to show how gauze is cut to make a basket   A B C and D —Dotted lines where cuts are made

The substratum of the culture is formed with washed sand which is too coarse to pass through the wire gauze   An alternative that can be used if sand does not happen to be available is small glass-beads, just too large to pass through the baskets, but the number of larvæ extracted from bead cultures is, on the whole, lower than from sand

A supply of earth is prepared by heating it in a moist state for twenty minutes at a temperature of 70°C , keeping the earth stirred during the process so that all of it is raised to a sufficiently high temperature to kill all the helminth eggs, larvæ and adult worms that it contains   The earth is now allowed to dry and is pounded lightly in a mortar   after which it is passed through a sieve with a mesh of about 3 mm   square , this provides a quantity of evenly divided earth that is in small enough particles to allow of its being accurately measured, and at the same time

ensures all the larvæ subsequently grown in it being under comparable conditions regarding the physical state of the soil.   This earth should be tested for the presence of helminth larvæ by putting up a culture of earth alone, and extracting it in the usual way, before it is employed for growing hookworm cultures.   If a large stock of earth has been prepared and it is kept for some time it should be periodically tested in the same way to see that it remains free of worms.

The contents of one of the baskets made of the dimensions given above is 108 c.c., a convenient amount of sand to use in a basket of this size is about 50 c.c.   A test-tube marked at the correct height is a suitable measure.   Such a quantity of sand provides a layer in the basket about 1.5 cm. in thickness, and a shallow depression is made in the centre of the sand taking care that it is not deep enough to expose the gauze at the bottom of the basket.   About 8 c.c. of prepared earth is now taken, using any small tube as a measure.   The actual quantity of earth used is not of much importance so long as exactly the same amount is always used for any series of cultures that are to be compared.

The description given is the exact account of how the cultures, the results of which are given in Tables I and II, were dealt with.   This is of course capable of general application in the use of the method.

In the cultures, either 22 c.c. or 44 c.c. of sand or beads were used as a substratum, and from the results it is clear that this degree of difference in volume has no constant bearing on the number of larvæ subsequently extracted.   This variation was made as it was thought possible that the greater the total volume of the culture the fewer larvæ would be extracted.

No estimation of the numbers of eggs in the various stools employed were made, because it has already been shown by Maplestone (1924) that this is an efficient method of growing hookworm larvæ, for it was used on that occasion to check the value of Stoll's egg-counting method, and many times more larvæ were extracted from cultures than one was led to expect from the number of eggs estimated by counting.

Eight c.c. of prepared earth were placed in the shallow depression in the substratum, and 4.4 c.c. of diluted stool were poured over the earth.   As stated above, no egg counts were made before making the cultures, and the stools were well stirred with an unmeasured amount of water, the amount varying with the original consistency of the different stools employed, but they were all brought to approximately the same state of semi-fluidity.   Four cultures were put up from the same diluted stool in each series, and the comparison of the four results served as a cross check.   The reason that the stools were broken up in water is because Heydon (1927) showed that stools treated in this way produced more larvæ than non-comminuted lumps of formed stool of the same size.   The reason for this is that eggs in the centre of a stiff lump of fæces have very little chance of developing and hatching successfully, compared with eggs near the surface of the stool, and comminution in water has the effect of bringing all the eggs relatively near the surface of the fæces, when it is spread in a thin layer on the earth.   Dilution in this way also has the advantage of rendering measurement of equal parts of a stool rapid and accurate.   A pipette with a wide opening and fitted with a rubber-teat is used for measuring the amount of stool.   It is marked at a suitable level and a

PLATE XIV.
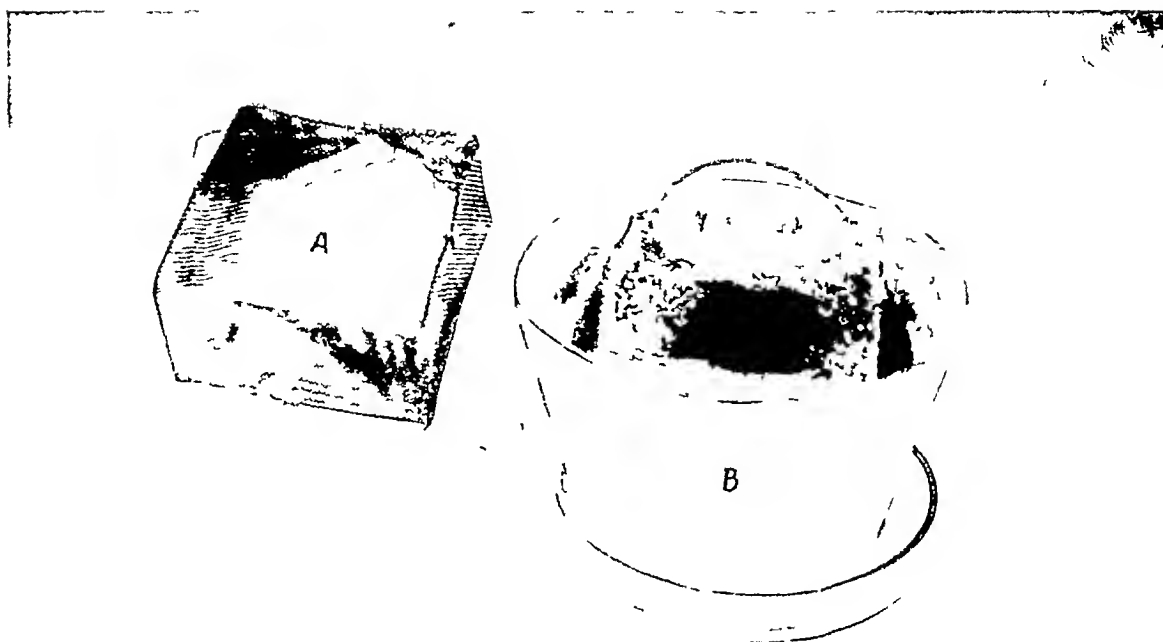
Fig 1   A —Wire basket   B —Culture in holder standing in Petri dish
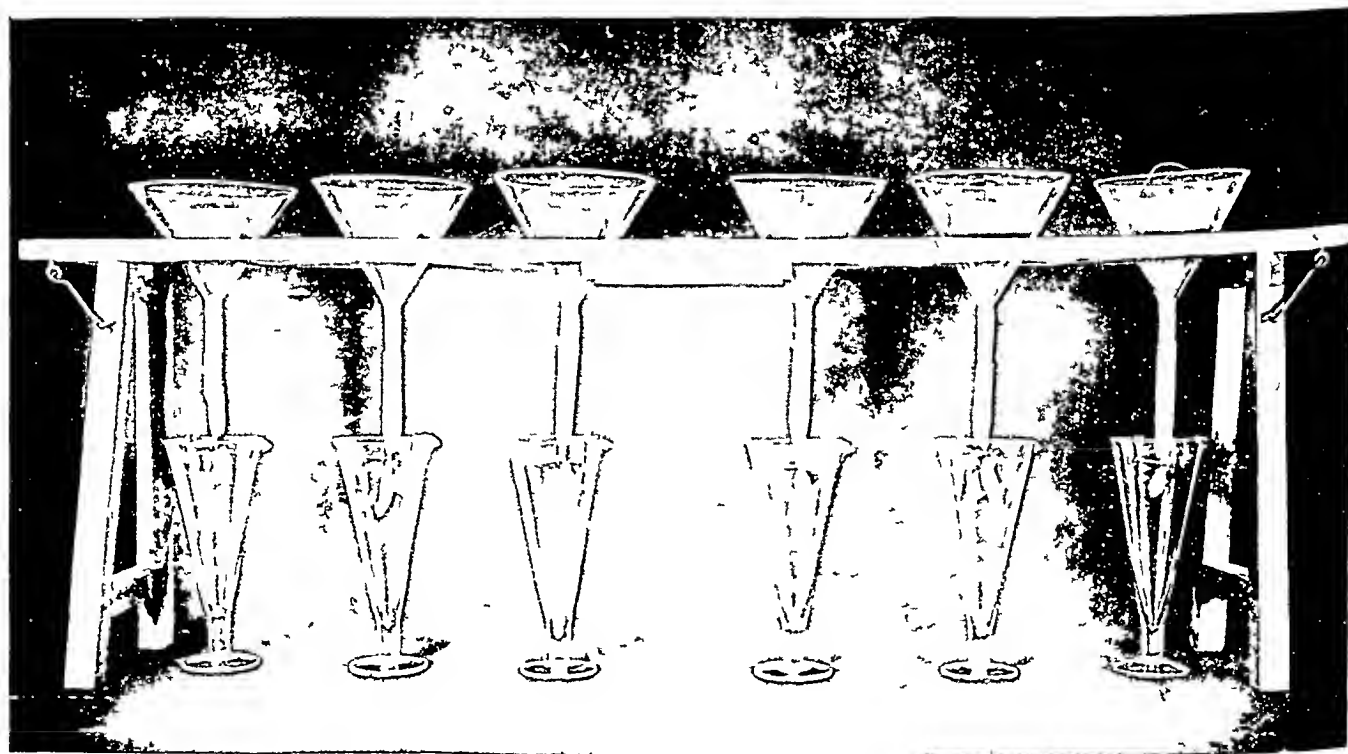
Fig 2   Folding funnel stand with extraction apparatus in position

portion of the diluted fæces is drawn into it up to this mark, and it is then expelled on to the earth in the culture basket   This operation is repeated until the desired amount of stool is added to the culture, and then the tube is washed by drawing water into it and expelling this on to the culture as well   Here again the actual amount of stool employed in any series of cultures is not of great importance so long as the same amount is taken for all the cultures in a series   But if it is desired to know exactly how much stool has been used it is easily ascertained, by drawing water into the pipette up to the mark on it and expelling this into a graduated tube

The cultures were then placed in small holders especially constructed for the purpose   At first tin was used for this purpose, but this rusts after being used for some time, with the result that all the apparatus becomes more or less soiled, so aluminium was substituted and this objection thereby overcome   These holders are truncated cones open at both ends, 10 cm in diameter at the large end, 6 5 cm at the small end, and about 8 cm in height   The diagonal measurement of one of the wire baskets is about 9 cm so that when placed inside the cone, at the large end, it rests against the sides by its four corners only, and about 3 cm from the top   This is now placed in a Petri dish containing a little water and put under a bell-jar (Plate XIV, fig 1)   Four such cultures can be accommodated under a bell-jar 30 cm in diameter, and the evaporation of water from the four Petri dishes helps to keep the air beneath the jar fairly moist so that evaporation from the cultures is slight , the result is that they only need a very small addition of water during the period of growth, and the larvæ are, therefore, in an evenly moistened medium during the whole week or ten days they are growing   In the third part of this paper discussion on the migration of larvæ from this type of culture is given, but it may be mentioned here that at odd intervals the water in the Petri dishes in which the cultures have been standing has often been examined and no larvæ have ever been found in it

After growing for the desired time (in the present instance this was one week) the cultures are extracted after the well-known method of Baermann with the modification introduced by Cort and his co-workers (1922)   For the sake of completeness a brief description of the apparatus may be given with advantage —

Glass-funnels of a suitable size fitted with a piece of rubber-tube closed by a clip and any convenient funnel stand are all that is needed   Plate XIV, fig 2, shows a very useful type of stand, which we had constructed to our own design, and which can be easily made by any carpenter   The whole stand can of course be made rigid, but we have found that the folding type illustrated is much more convenient as it can be packed away when not in use   Reference to the figure will show how it can be made so that it will fold   The legs are hinged on the top board and when in use they are held in position by hooks and eyelets   The board on the top is also hinged in the centre so that it folds into half its length for packing

A culture is simply lifted by its handle and the basket transferred to the funnel, within which it stands on its four corners   Originally, when only earth was used it was necessary to place a piece of cloth inside the funnel before placing the culture in it , this was to prevent fine particles of earth falling through the wire gauze along

with the larvæ and settling at the bottom of the clamped rubber-tube   The presence of earth at this stage of the process is a great disadvantage as it renders counting of larvæ very difficult and tedious   Usually, even when calico was used a certain amount of earth managed to percolate through, and the extracted larvæ were rarely entirely free from it   Another possible objection to the use of cloth is that larvæ may become entangled in it or migrate upwards through the saturated fibres and so not reach the bottom of the water in the funnel   It must be admitted that we have never succeeded in extracting larvæ from calico that has already been used for a culture, but we do not consider the proof that they are not present to be fully established   The use of coarse sand or beads as a basis for the cultures entirely prevents earth from percolating through into the funnel, and so calico need not be used   This simplifies the process of extraction considerably, and also does away with the possible objection to calico, mentioned above

After a culture basket is placed in a funnel, water heated to 37°C is poured into it care being taken to run it down the side of the funnel so as not to disturb the culture   The water is poured into the funnel until it comes into contact with the culture from below, but the culture is not completely submerged   For the size of culture under discussion about 220 c c to 250 c c of water is needed   The culture is then left until the next morning before the water is drawn off, that is, a period of eighteen to twenty hours   When the water is to be drawn off the clamp is partially released and about 15 c c allowed to flow into a graduated centrifuge tube   After this the clamp is removed altogether and the remainder of the water allowed to run into a conical urine test glass

The centrifuge tube is given a few spins in the machine (half a minute at 1,000 r p m), which are sufficient to throw all the larvæ to the bottom of the tube   The supernatant water is pipetted off until 5 c c are left   An even suspension of the larvæ is now made by drawing up and rapidly expelling back into the tube with a pipette some of the water , this operation is repeated several times   It can be seen easily with the naked eye when the larvæ are evenly distributed throughout the water   A graduated pipette is used and when the mixing is complete 0 1 c c of suspension is drawn up and transferred to a microscope slide   This is repeated five times, care being taken to re-agitate the suspension of larvæ on each occasion, because they settle very rapidly   The number of larvæ on each slide is counted under the low power of the microscope without a cover-slip   It greatly facilitates counting if the larvæ are made immobile and this is easily done by adding a drop of Lugol's iodine solution to the water on the slides   An eyepiece with a square aperture and a mechanical stage are needed, so that the whole of the drop is accurately reviewed without any overlapping   When only part of a larva appears in the field one should only count those appearing either on the top or bottom edge, depending on whether the counting was commenced at the upper or lower edge of the drop of water   The total number of larvæ in the five drops added together and multiplied by ten gives an accurate estimate of the total number of larvæ in the tube   If a culture happens to be very prolific it may be found difficult to count all the larvæ in a sample of 5 c c suspension, in which case it may be necessary to dilute it to 10 c c or even more, before taking the 0 1 c c samples, and then of course the basis of calculating the total number of larvæ is altered accordingly   The balance of the water which has been standing meanwhile in the test glass is now carefully siphoned off until about

15 c c to 20 c c is left   It is transferred to a centrifuge tube with a pipette, and care is taken to agitate it well so that no contained larvæ will be left behind   It is now centrifuged and any larvæ it may contain added to the total already obtained This is an added precaution that renders the results as free from error as possible, but in our experience the danger of larvæ remaining in the residual water of the extraction apparatus is so slight as to be almost negligible, for we rarely found any larvæ at all in this second portion of the water, and on the few occasions that they have been present they have never amounted to a total of one per cent of the total number extracted

MIGRATION OF LARVÆ FROM CULTURES

On account of the climatic conditions in Calcutta we are enabled to grow our cultures at room temperature   There is thus the normal diurnal variation, and as Lane (1928) made his observations with cultures in an unvarying incubator temperature it was thought possible that this abnormal factor might be responsible for the migration of larvæ   Accordingly, cultures were at first put up exactly as Lane had done, with the sole exception that the Petri dishes we used were 90 mm in diameter, whereas Lane used dishes of 48 mm , and even with the larger dishes irregular numbers of larvæ migrated just as Lane had described

We now took cultures of the size and type described in the first part of this paper, and placed them in funnels fitted with clamped rubber-tubes, such as are used for extraction   Water was poured into the funnel until it was within two or three millimetres of the bottom of the wire baskets, and they were left thus for the period of growth (7 to 10 days)   Larvæ were never found in the water in these funnels, and the cultures always produced numerous healthy and active larvæ, when extracted after the manner described above   This experiment was performed on over fifty cultures and as the results were uniformly negative regarding the water in the funnels below the cultures it is not considered necessary to give the actual detail of the results in figures

Although these findings appeared conclusive, it was decided to test more severely whether larvæ would migrate from this type of culture   For this purpose much smaller baskets measuring only 3 cm square and about 2 5 cm in depth were made, and these were filled with earth and stool placed on the surface in the usual way   They were then suspended by their handles on a glass-rod passing across the top of a beaker in which there was water just below the bottom of the baskets   It was thought possible that the larvæ might more readily leave cultures in which they only had a maximum distance of 1 5 cm to travel to reach the edge of the culture from its centre   Table I shows that, although the migration of larvæ was not as universally negative as in the case of the larger cultures, the remarkably small number leaving the cultures indicates that their departure was more a matter of chance than of purposive action

It will be noted that six of the cultures were in pure earth, and the remainder in a mixture of earth and sand in equal parts   This variation was introduced to find out if a marked difference in migratory habits was exhibited in earths of distinctly different physical character   As there was no apparent difference in these two sets of cultures further variations in the earth medium were not made

Table I

Migration of larvæ from small cultures

Number		Water in beakers below cultures	Larvæ extracted from cultures
Pure earth	1	0	982
	2	1 larva	925
	3	0	700
	4	0	396
	5	1 larva	600
	6	1 larva	814
Sand and earth	1	0	277
	2	0	382
	3	14 larvæ*	735
	4	0	408
	5	1 larva	479
	6	12 larvæ*	473

* When moistening the cultures it was noted on these two occasions that excess of water had been accidentally added, and a few drops fell from the culture into the water below, and these drops probably carried the larvæ with them In none of the other cultures was more water added at any time than the earth could absorb without overflow

Another experiment was performed to test still further if hookworm larvæ showed any tendency to leave suitably moistened earth and enter water for preference Three circular wire baskets were made 140, 100 and 53 mm in diameter respectively The largest one just fitted a Petri dish , the 100 mm basket was then placed in the centre of this, the 53 mm basket put in the centre of the whole, and the complete system was filled with helminth-free earth This Petri dish was

placed in the middle of a larger one which was 220 mm in diameter, and which contained water forming a moat in which to trap larvæ, after the method of Lane A portion of emulsified stool containing hookworm eggs was poured in the centre of the earth and the culture was kept under a bell jar from seven to ten days for different cultures, and the whole of the earth was kept in a suitable state of moistness throughout The small centre basket was lifted out and the contents transferred to a wire basket and extracted in the usual way The 100 mm basket was then removed and treated in the same way This basket contained a border of earth about 23 mm in width that had surrounded the central basket Finally, the third portion of earth which was in the 140 mm basket, was also extracted, and the results are given below These are expressed as single numbers but they are the average obtained from several cultures In the central culture 9,266 larvæ were isolated, in the 100 mm basket 196 larvæ, neither in the 140 mm basket nor in the water surrounding the cultures were any larvæ found The indication from this series of observations is that only about 2 per cent of larvæ wandered more than $26\frac{1}{2}$ mm from the centre and the most energetic of these was not found more than a possible maximum of 50 mm from the point where it had hatched out

EXTRACTION RESULTS

These figures were obtained from extraction of the culture the detailed preparation of which has already been given under 'method of constructing the apparatus'

Table IIa shows the results when four cultures from each stool were put up, in each instance two cultures being put up with 22 c c of beads as a base, and two cultures with 44 c c of beads as a base

Table IIb shows the results when four cultures were also made from each stool used but beads were used as a base for two cultures and sand for the other two cultures in each series the differences being that 22 c c of beads or sand and 44 c c of beads or sand were used according to the headings given in the table

Although care was taken that each series of cultures contained equal amounts of stool the total number of larvæ extracted showed considerable variation in many instances This may be due to unequal distribution of eggs in the emulsified fæces, failure of a certain number of eggs to hatch, or failure of larvæ to survive until the time of extraction Whatever the cause of this discrepancy may be, it is clear that single cultures are quite unreliable for estimating, by the larvæ extracted, how many eggs the stool contains

It is also clear that a single extraction is liable to give uncertain results regarding the total number of larvæ in a culture but that if the numbers extracted on the first two days are added together one can reasonably expect that 95 per cent of the total number of larvæ produced will be recovered To give our results more exactly, 95 per cent of the larvæ were extracted in 78 per cent of instances, or in the whole series 96 per cent of larvæ were extracted on the first two days

Serial number of cultures	BEADS 22 CC																	
	1st Culture									2nd Culture								
	Days of extraction							Percentage of larvæ extracted on		Days of extraction							Percentage of larvæ extracted on	
	1	2	3	4	5	6	7	1st day	1st & 2nd days	1	2	3	4	5	6	7	1st day	1st & 2nd days
1	440	46	41	6	0			82 5	91 3	562	21	4	2	0			95 4	98 9
2	179	13	4	0				91 3	98 0	258	9	6	4	0			93 1	96 2
3	467	26	6	0				93 5	9C 7	518	19	6	0				95 4	98 8
4	108	55	7	2	0			86 4	97 4	302	12	0					96 1	100 0
5	157	27	14	1	0			78 9	92 5	127	38	23	26	2	1	0	58 5	76 0
6	898	50	6	5	0	1	0	93 5	98 7	875	51	5	3	2	0		93 4	98 9
7	33	16	3	1	0			61 1	92 6	482	69	15	4	0	1	0	84 4	96 5
8	785	81	12	6	0			88 8	97 8	500	52	44	16	27	10	6	76 3	84 2
9	205	23	15	1	1	0		83 6	93 0	201	17	0	1	0			91 8	99 5
10	177	1	1	0				98 8	99 4	189	26	2	1	0			86 7	98 6
11	129	24	0	1	0			83 7	99 3	191	10	4	0				93 1	98 1

Serial number of cultures	SAND 22 CC									BEADS 22 CC								
	1st Culture									2nd Culture								
	Days of extraction							Percentage of larvæ extracted on		Days of extraction							Percentage of larvæ extracted on	
	1	2	3	4	5	6	7	1st day	1st & 2nd days	1	2	3	4	5	6	7	1st day	1st & 2nd days
12	3,367	460	127	42	5	0		82 1	90 9	3,041	549	149	25	12	5		76 4	90 1
13	869	79	15	0				90 2	98 4	711	41	19	1	1	0		91 9	97 2
14	211	47	4	0				80 5	98 4	94	19	1	1	0			81 7	98 5
15	1,045	147	39	6	4	2	0	84 0	87 8	482	36	6	3	2	1	0	90 1	97 7
16	630	41	16	6	4	0		90 3	96 2	560	20	0					96 7	100 0
17	782	295	79	28	10	4	3	65 2	90 5	653	96	20	4	1	0		84 6	96 6

IIa

BEADS 44 CC																	
3RD CULTURE										4TH CULTURE							
Days of extraction							Percentage of larvæ extracted on		Days of extraction							Percentage of larvæ extracted on	
1	2	3	4	5	6	7	1st day	1st & 2nd days	1	2	3	4	5	6	7	1st day	1st & 2nd days
460	8	3	0				97 6	99 1	120	15	7	7	0			93 4	96 8
303	19	6	1	0			92 0	97 8	284	15	7	0				92 8	97 7
516	33	10	4	0			91 6	97 5	473	54	17	0				86 9	96 8
242	6	1	0				97 1	99 6	226	15	3	2	0			91 8	97 9
164	11	13	5	3	0		83 6	89 2	161	21	13	9	0			78 9	89 2
806	48	10	2	2	3	0	92 5	98 0	798	94	5	7	3	0		87 9	98 2
232	13	5	5	1	0		90 2	95 5	36	9	8	5	0			62 0	77 6
530	26	19	3	2	0		90 1	95 8	649	41	70	8	3	0		84 2	89 5
275	13	2	5	0			93 2	97 6	287	26	1	2	0			90 8	99 0
223	15	3	1	0			92 1	98 3	177	7	3	0				94 4	98 4
141	34	3	0				79 2	98 3	154	41	1	1	0			78 1	98 9

IIb

SAND 44 CC									BEADS 44 CC								
3RD CULTURE									4TH CULTURE								
Days of extraction							Percentage of larvæ extracted on		Days of extraction							Percentage of larvæ extracted on	
1	2	3	4	5	6	7	1st day	1st & 2nd days	1	2	3	4	5	6	7	1st day	1st & 2nd days
4,700	169	92	30	6	3	5	93 9	97 2	2,860	266	39	31	6	10	4	87 8	96 0
712	69	35	1	1	0		87 1	95 4	796	41	13	1	0			93 5	98 3
133	26	4	0				81 6	97 6	110	16	9	0				85 9	93 3
934	75	14	3	3	1		90 6	97 9	777	56	15	3	0			91 3	97 8
971	71	21	5	0			90 9	97 5	684	25	5	0				95 7	99 3
1,049	88	31	7	1	0		89 2	97 3	846	95	5	4	0			89 0	99 0

CONCLUSION

A simple method of culturing hookworm larvæ is described, and it has been shown that larvæ will not migrate from cultures of the type described   It is also shown that it is necessary to extract cultures for at least two successive days to be sure that nearly all the larvæ in the cultures are recovered

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STUDIES ON VITAMIN B$_1$

Part I

SOLUBILITY OF THE VITAMIN AS PRESENT IN THE INTERNATIONAL STANDARD PREPARATION

BY

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THE present paper deals with certain observations made by us in regard to (*i*) the relation of the solubility of vitamin B$_1$ (as present in the International Standard Preparation) to pH , (*ii*) the relation between pH and λ-maximum (i e , wave-length at which absorption is maximum) , and (*iii*) the means to be adopted for obtaining standard solutions of the vitamin, from the International Standard Preparation (Kaolin adsorbate), at different levels of pH

This Preparation is made from watery extracts of rice-polishings acidified with sulphuric acid , from these extracts the vitamin is adsorbed on Kaolin at pH 4 5 (Jansen and Donath, 1927)   For our purpose extracts of this Preparation were made with water of varying pH , the solution thus obtained being subjected to spectrographic examination in order to determine the amount of the vitamin that had gone into solution   This method of determination is based on the observations of Bowden and Snow (1932), Heyroth and Loofbourow (1932) and others that the absorption maximum of light at wave-length 2600 A U is specific for vitamin B$_1$ And, further, that extinction coefficients (i e , degrees of absorption) for this wave-length, observed with different preparations of vitamin B$_1$, are proportional to the biological activities of the preparations, i e , to their vitamin B$_1$ contents

Procedure

Known weights of the International Standard (I S) Preparation of vitamin B$_1$ were treated with 5 c c buffer (in water) of known pH   The mixtures were thoroughly stirred with a glass-rod for five minutes and then centrifuged, the supernatant fluid being used for spectrophotometric observation   The addition of the

I S Preparation to the buffer did not alter its pH as tested by the quinhydrone method During the progress of the work it was observed that the lower the pH the more I S Preparation had to be taken in order to get the absorption curve within the range of the instrument, the higher the pH the less I S Preparation had to be taken Thus 125 mg were needed at pH 1 2, while 10 mg sufficed at pH 12 36 (Table I) Citrate and borate mixtures (Clark, 1920) were used as buffers Phthalate (Clark, 1920) could not be used as it showed marked absorption in the ultra-violet Hilger's Rotating Sector, in conjunction with a medium-sized Quartz Spectrograph (Hilger's E316 with internal wave-length scale), was used The source of the ultra-violet light was a spark between steel-tungsten electrodes with a gap of 4 mm ; the voltage was 15,000 The slit width was 0 025 mm , the initial exposure 10 seconds The liquid containers were 1 cm quartz cells.

Results.

Nine observations were made over a range of pH from 1 2 to 12 36 The absorption curves for all of these have been drawn , extinction coefficients being shown as the ordinates and wave-lengths as the abscissa A sufficient number of these curves for illustrative purposes is shown in Figs 1 to 5
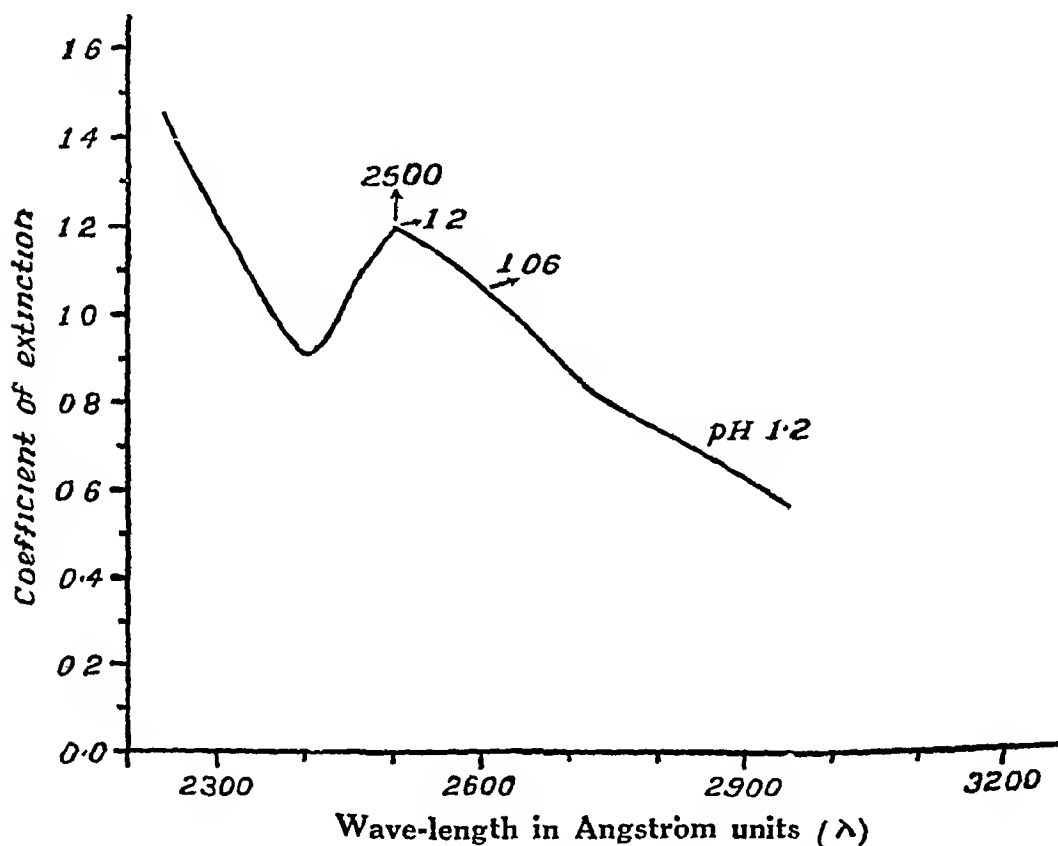
Fig 1 Absorption curve of vitamin B_1 solution obtained from 125 mg International Standard Preparation in 5 c c buffer at pH 1 2 Maximum absorption occurred at 2500 A U , extinction coefficient for wave-length 2600=1 06
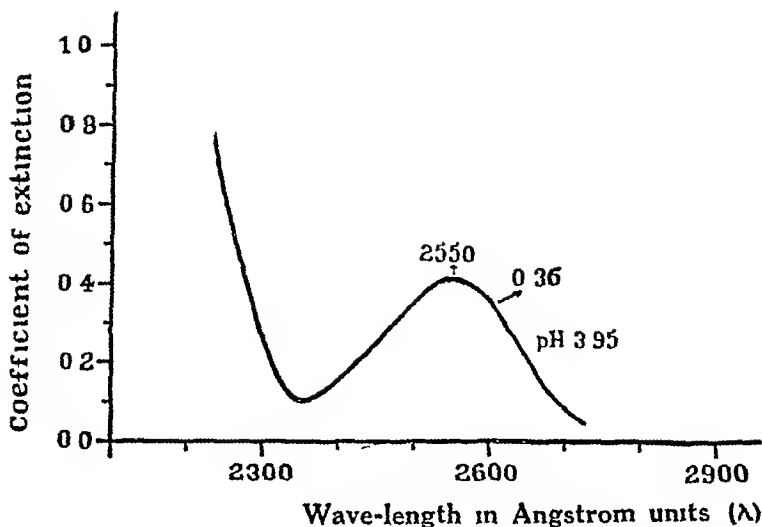
Fig 2   Absorption curve of vitamin B_1 solution obtained from 75 mg.
International Standard Preparation in 5 c c buffer at pH 3 95   Maximum
absorption occurred at 2550 A U , extinction coefficient for wave length
2600=0 36
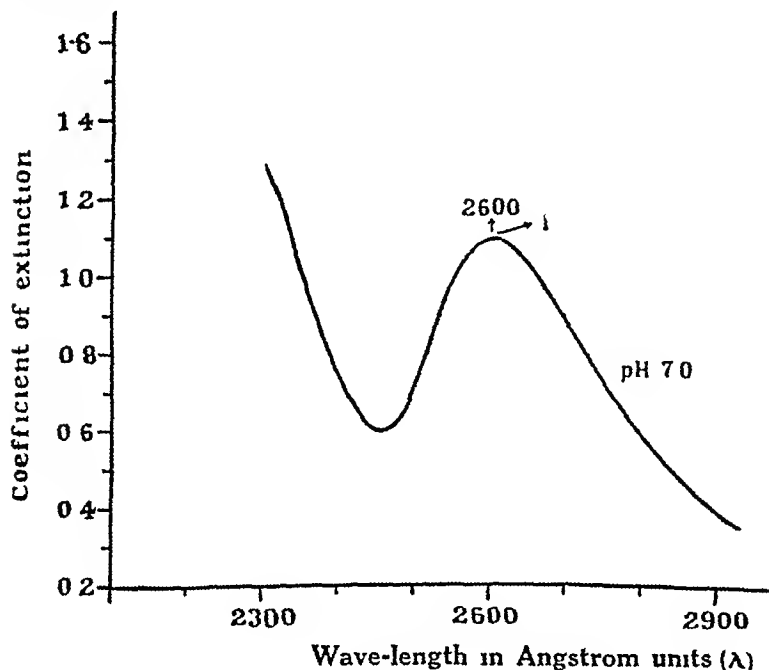
Fig 3   Absorption curve of vitamin B_1 solution obtained from 20 mg
International Standard Preparation in 5 c c buffer at pH 7 0   Maximum
absorption occurred at 2600 A U , extinction coefficient for wave length
2600=1 10
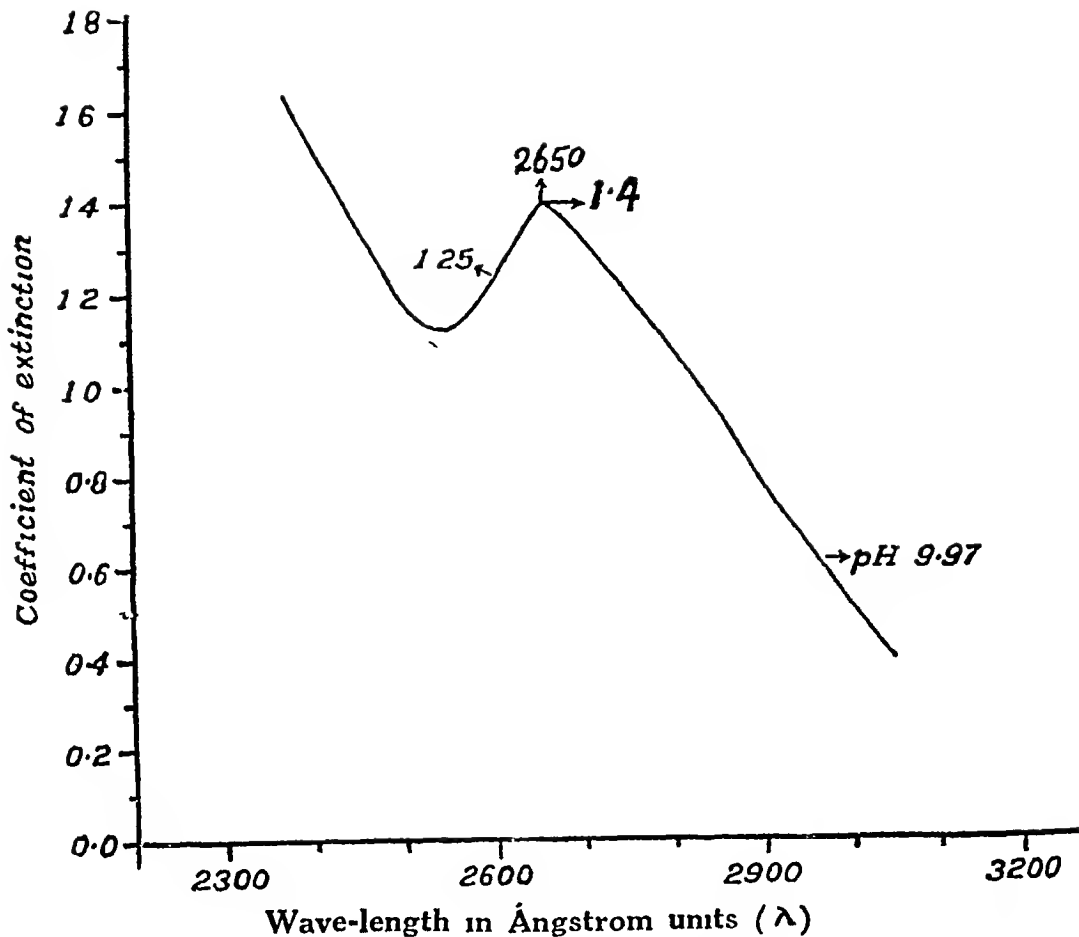
Fig 4   Absorption curve of vitamin B₁ solution obtained from 15 mg International
Standard Preparation in 5 c c buffer at pH 9 97   Maximum absorption occurred at 2650
A U , extinction coefficient for wave length 2600=1 25

Fig 5   Absorption curve of vitamin B₁ solution obtained from 10 mg International
Standard Preparation in 5 c c buffer at pH 12 36   Maximum absorption occurred at 2680
A U , extinction coefficient for wave length 2600=1 00

Tables I and II give λ-maximum at different levels of pH and the amount of the I S Preparation of vitamin B_1 that was treated with 5 c c buffer in each case In order to compare the observations at different levels of pH, the figures given in column 6 of both Tables, from which such comparison can be made, were calculated from the observed data as set out in column 5 of the respective Tables

TABLE I

Giving the data of nine spectrographic observations made at levels of pH ranging from 1 2 to 12 36

Number of obser vation	pH	Wave length at which maximum absorption occurred	Amount of the I S Prepara tion ex tracted with 5 c c buffer, in mg	Observed extinction coefficient for wave length 2600	Calculated extinction coefficient for 100 mg I S Prepa ration in 5 c o buffer at wave length 2600=solubi lity of the vitamin
(1)	(2)	(3)	(4)	(5)	(6)
1	1 20	2500	125	1 06	0 85
2	2 97	2540	125	0 56	0 45
3	3 95	2550	75	0 36	0 48
4	4 96	2570	50	1 38	2 76
5	5 80	2590	20	0 69	3 45
6	7 00	2600	20	1 10	5 50
7	8 91	2620	20	1 50	7 50
8	9 97	2650	15	1 25	8 33
9	12 36	2680	10	1 00	10 00

Studies on Vitamin B₁.

TABLE II

*Giving the data of nine spectrographic observations made at
levels of pH ranging from 1 2 to 12 36*

Number of obser vation	pH	Wave length at which maximum absorption occurred	Amount of the I S Preparation extracted with 5 c c buffer, in mg	Observed extinction coefficient for wave lengths at which maximum absorption occurred	Calculated extinction coefficient for 100 mg I S Preparation in 5 c c buffer at λ-maxima= solubility of the vitamin
(1)	(2)	(3)	(4)	(5)	(6)
1	1 20	2500	125	1 2	0 96
2	2 97	2540	125	0 6	0 4S
3	3 95	2550	75	0 4	0 533
4	4 96	2570	50	1 44	2 88
5	5 8	2590	20	0 7	3 50
6	7 00	2600	20	1 1	5 50
7	8 91	2620	20	1 52	7 60
8	9 97	2650	15	1 4	9 33
9	12 36	2680	10	1 12	11 20

Tables I and II represent different methods of arriving at the extinction co-efficients that would be given by 100 mg of the I S Preparation at the different levels of pH   In the first Table the calculation in each of the nine observations is made for a wave-length of 2600   In the second Table it is made for the wave-length at which maximum absorption occurred   this wave-length varying from 2500 at pH 1 2 to 2680 at pH 12 36   It is difficult, in the present state of know-ledge, to decide which is the more accurate procedure , we have accordingly given the

results arrived at by following both procedures It will be noted from a comparison of the figures given in column 6 of Table I with those in column 6 in Table II, and of the curves (Figs 7 and 8) illustrating them, that there is no great difference between the two sets of figures The differences are set out in Table III —

TABLE III.

Giving the calculated extinction coefficients for 100 mg of the International Standard Preparation in 5 c c buffer at wave-length 2600 and at the wave-lengths at which maximum absorption occurred

Number of obser vation	pH	Extinction coefficient at A U 2600	Extinction coefficient at wave-lengths at which maximum absorption occurred
(1)	(2)	(3)	(4)
1	1 20	0 85	0 96
2	2 97	0 45	0 48
3	3 95	0 48	0 53
4	4 96	2 76	2 88
5	5 80	3 45	3 50
6	7 00	5 50	5 50
7	8 91	7 50	7 60
8	9 97	8 33	9 33
9	12 36	10 00	11 20

Three curves have been drawn from the data given in Tables I and II   (*i*) pH versus λ-maximum (Fig 6) , and (*ii*) Solubility versus pH (Figs 7 and 8)

pH versus λ-maximum (Fig 6) —An examination of Fig 6 shows that with increase of pH (i e , decrease of H-ion concentration) the absorption band shifts towards the longer wave-length and vice versa   Smith (1928) in his absorption work with uric acid noted similar shifts of λ-maximum , so also did Holiday (1930) in his work with guanine
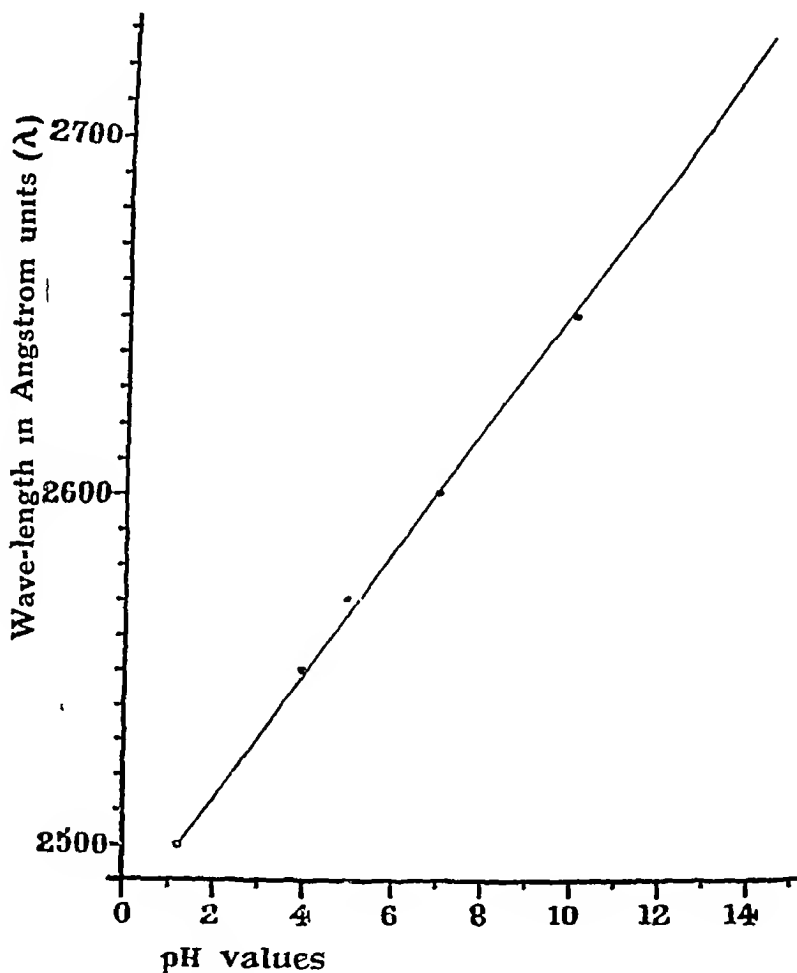
Fig 6   Showing the relationship between shift in wave length maximum and pH (columns 2 and 3, Table I)

But their observations were not made over the whole range of pH and their data do not show any definite relation between the shifts of λ-maximum and the change in pH   The curve in Fig 6 is of a definite character   it seems to be a straight line and to indicate that the λ-maximum for vitamin B_1 shifts in proportion to the change in pH   So far as we are aware no mention of this shift in

wave-length has hitherto been made by workers employing spectrographic methods for the study of vitamin B_1

Solubility versus *pH* (Figs 7 and 8) —Since the extinction coefficients, i e , degrees of absorption, will be proportional to the quantity of the vitamin that has
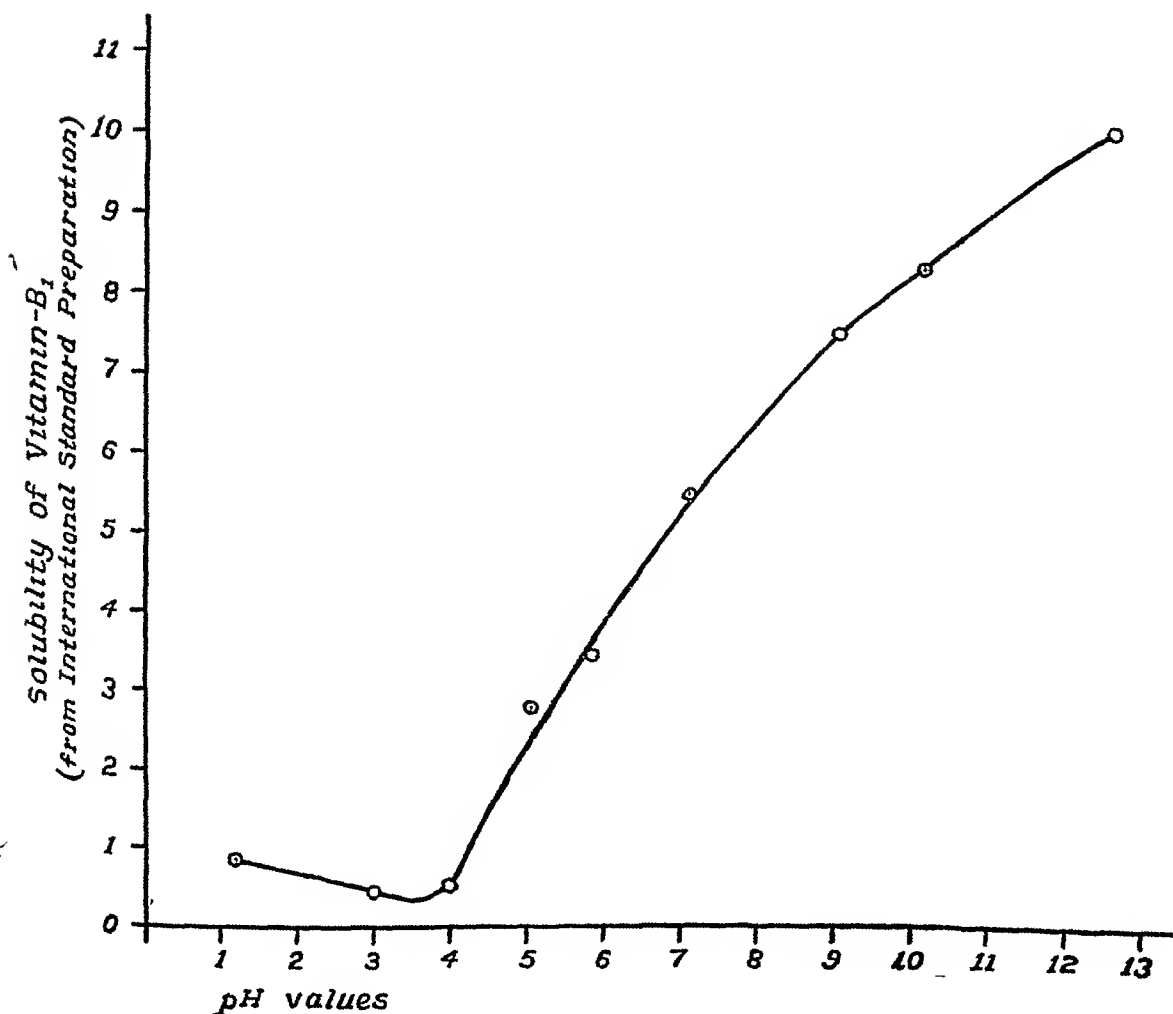
Fig 7   Showing the solubility of vitamin B_1 from International Standard Preparation at different pH-values
(columns 2 and 6, Table I)   Extinction coefficients calculated for 2600 A U

actually gone into solution, solubility is represented by the figures given in columns 6 of Tables I and II   From these figures it would seem that solubility of the vitamin (from the I S Preparation) is least between pH 3 and pH 4, that from

this zone it increases slightly with decreasing pH and increases markedly with increasing pH.
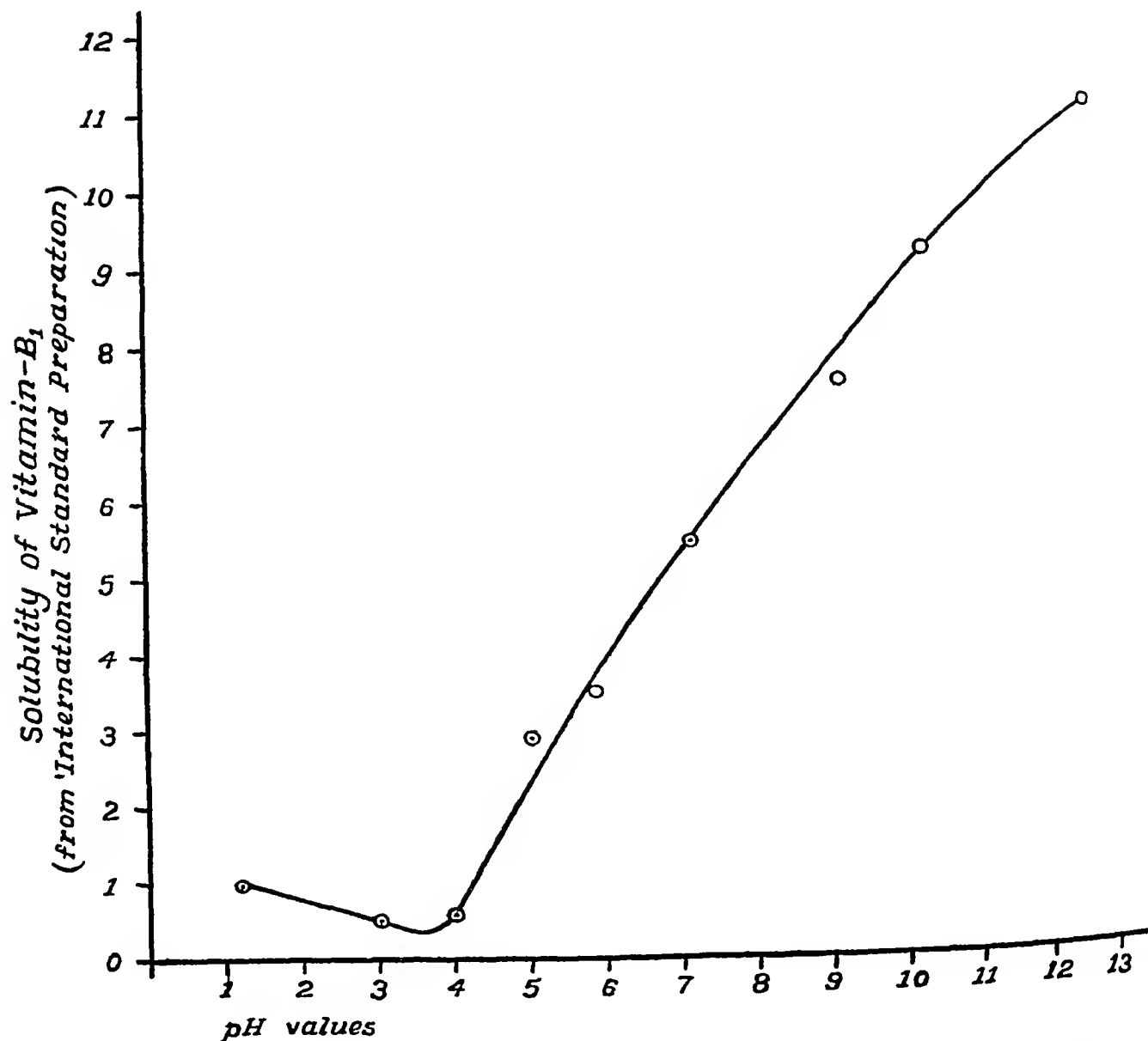
Fig 8 Showing the solubility of vitamin B₁ from International Standard Preparation at different pH values (columns 2 and 6, Table II)   Extinction coefficients calculated for wave lengths at which maximum absorption occurred   Note similarity between Figs 7 and 8

Salmon, Guerrant and Hays (1928), in their biological experiments with several preparations of the vitamin B-complex adsorbed on Fuller's earth, found that

optimal adsorption occurred at pH 1 decreasing rapidly with increasing alkalinity and slowly with increasing acidity. Our results are in conformity with those of these observers. Further, Kinnersley, O'Brien and Peters (1933), in preparing their crystalline product of vitamin B_1, have found that optimum crystallization takes place at pH 3. All these results suggest that the iso-electric point of vitamin B_1 lies between pH 3.0 and pH 4.0 and not above pH 8.5 as has been suggested by Birch and Guha (1931).

Standard Solution of Vitamin B_1 may be prepared from the Kaolin adsorbate (I. S. Preparation) in the way shown in Tables IV and V :—

<div align="center">TABLE IV</div>

pH	Extinction coefficient for one unit (10 mg) I S P in 5 c c medium, at A U 2600	Quantity of I S P (mg) needed to give the same concentration of vitamin B_1 at different levels of pH
1 20	0 085	64 71
2 97	0 045	122 22
3 53	0 032	171 87
3 95	0 048	114 59
4·96	0 276	19 92
5 80	0 345	15 94
7 00	0 550	10 00 The'International unit
8 91	0 750	7 33
9·97	0 833	6 60
12 36	1 000	5 50

Table IV has been prepared from the figures given in the last column of Table I, and Table V from those given in the last column of Table II. It will be noted that there is no great difference between them (*cf* Figs 9 and 10)

The International unit of vitamin B$_1$ was taken as the basis of the calculations This unit is defined[*] as the ' anti-neuritic activity of 10 milligrams of the International Standard Preparation '   It will be noted from Tables I and II that the co-efficient representing the amount of vitamin B$_1$ passing into solution from 10 units (100 mg) of the I S Preparation in 5 c c of water at pH 7 is 5 50 , it is, therefore,
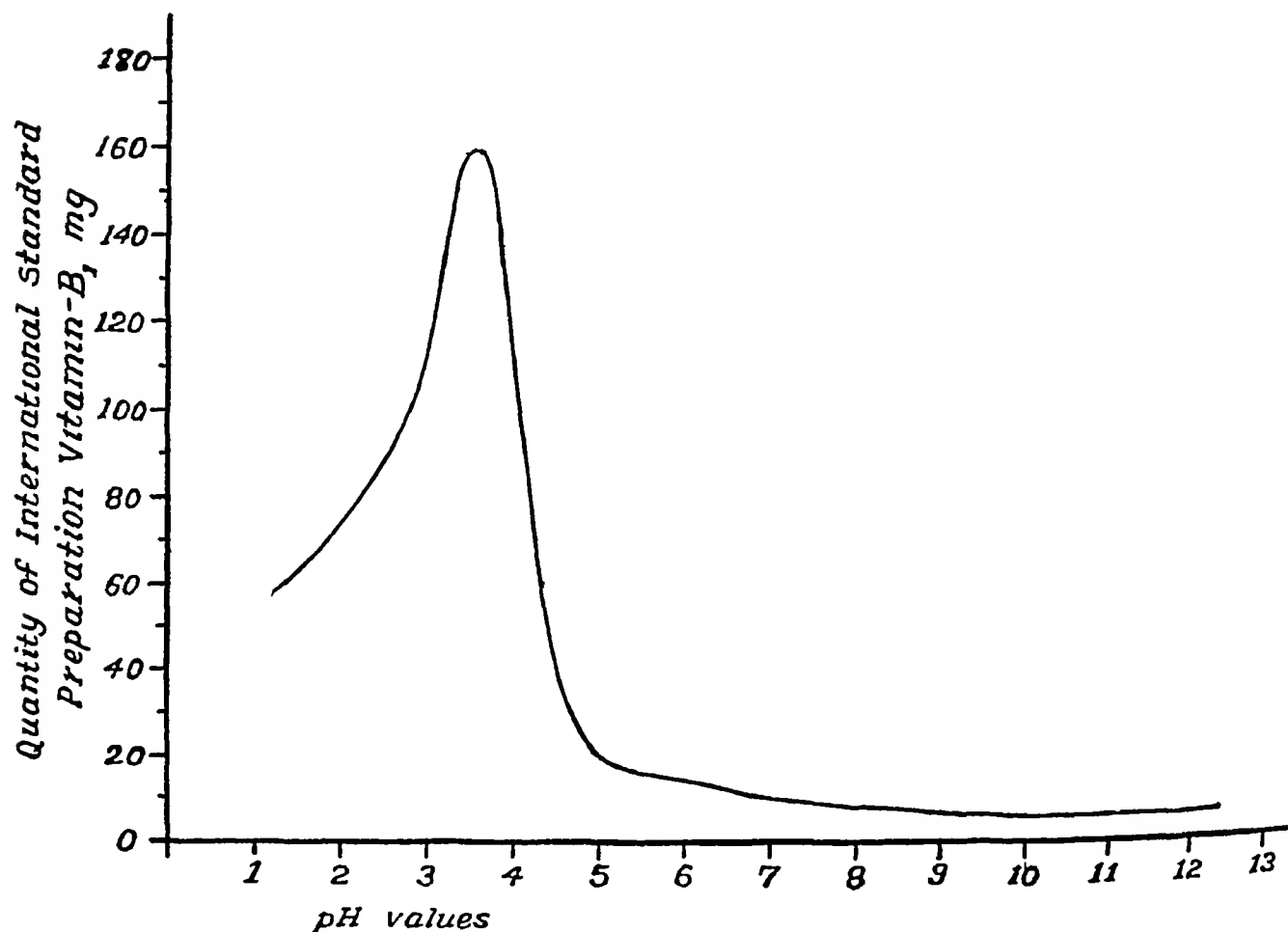
Fig 9   Quantity of International Standard Preparation of vitamin B$_1$ that has to be used at different levels of pH to give the same amount of vitamin B$_1$ in solution that is obtained with one International unit (i e , 10 mg) of the Preparation at pH 7 0 (*see* Table IV)

0 55 for one unit (10 mg) at this level of pH (approximately that of ordinary water) But the coefficient of solubility varies with pH , being as low as 0 032 to 0 034 per unit at pH 3 53 and as high as 1 0 to 1 12 at pH 12 36 (columns 2, Table IV and

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* League of Nations Health Organization (1932) Memorandum on the Standard Preparation of Anti neuritic Vitamin B$_1$

Table V) In order, then, to get the same concentration of the vitamin at different levels of pH as that in water at pH 7, more or less of the I S Preparation is needed according as the coefficient of solubility of the vitamin is lower or higher The actual quantities (Q) of the I S Preparation that are needed for this purpose at
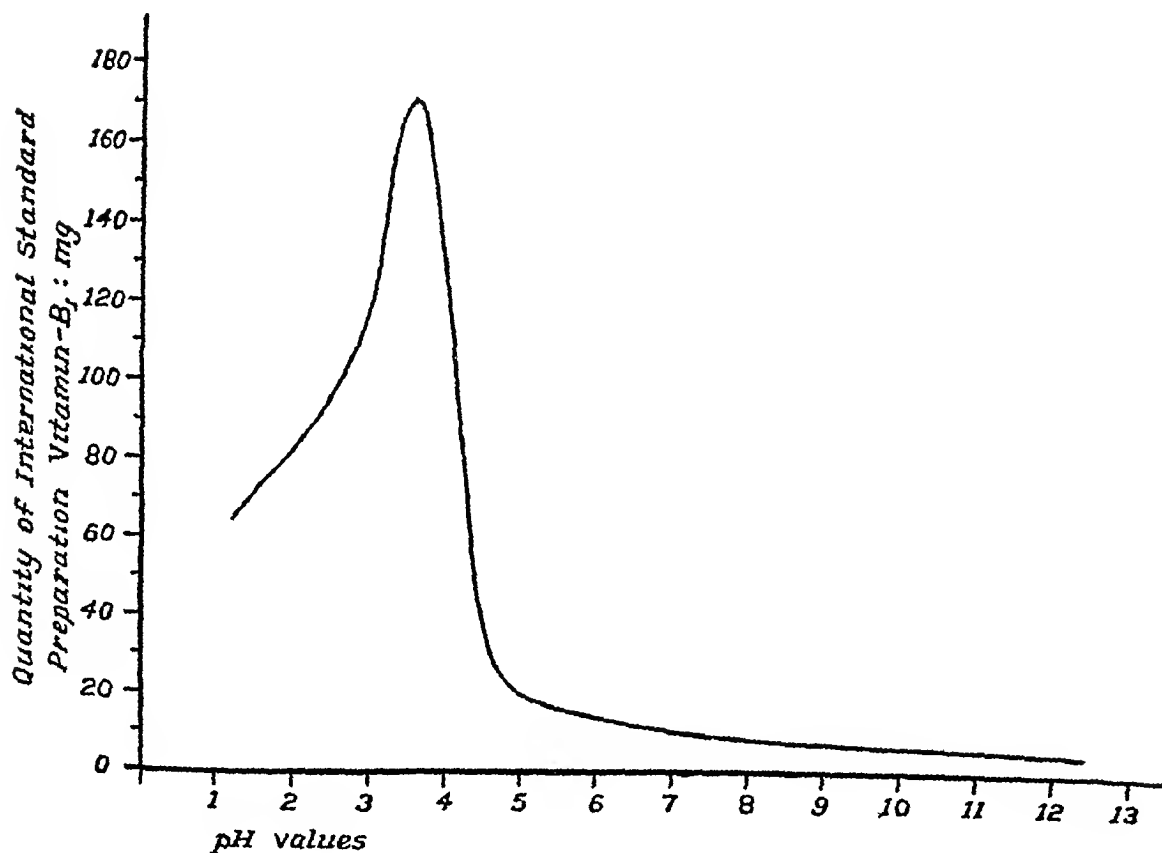
Fig 10 Quantity of International Standard Preparation of vitamin B_1 that has to be used at different levels of pH to give the same amount of vitamin B_1 in solution that is obtained with one International unit (i e , 10 mg) of the Preparation at pH 7 0 (*see* Table V)

different levels of pH are given in the third column of either Table IV or V according to which method of calculation is accepted They are arrived at by the formula $\frac{u \times 10}{c} = Q$, where u is the coefficient of solubility in water at pH 7 0 and c that at the given pH

In order to test the accuracy of these quantities, solutions of the vitamin in 5 c c. of medium were made from them at the levels of pH shown in column 1 of Table IV. These solutions were then subjected to spectrophotometric examination and absorp tion curves prepared for each   The similarity of all these curves was striking , five

Table V

pH	Extinction coefficient for one unit (10 mg I S P in 5 c c medium) at λ-maximum	Quantity of I S P (mg) needed to give the same con centration of vita min B₁ at different levels of pH
1 20	0 096	57 290
2 97	0 048	111 580
3 53	0 0343	160 349
3 95	0 0533	103 190
4·96	0 288	19 097
5 80	0 350	15 714
7 00	0 55	10 ᵒᵒ The Interna- tional_unit
8 91	0 76	7 237
9 97	0 93	5 914
12 36	1 12	4 910

of them (Figs 11, 12, 13, 14 and 15) are reproduced for illustrative purposes Moreover, the extinction coefficients at wave-length 2600 A U were in all cases practically the same , the amount of variation from that (0 55) of the solution at pH 7 being only ± 0 03   It is clear, therefore, that the figures given in the third column of Table IV are approximately correct

PLATE XV
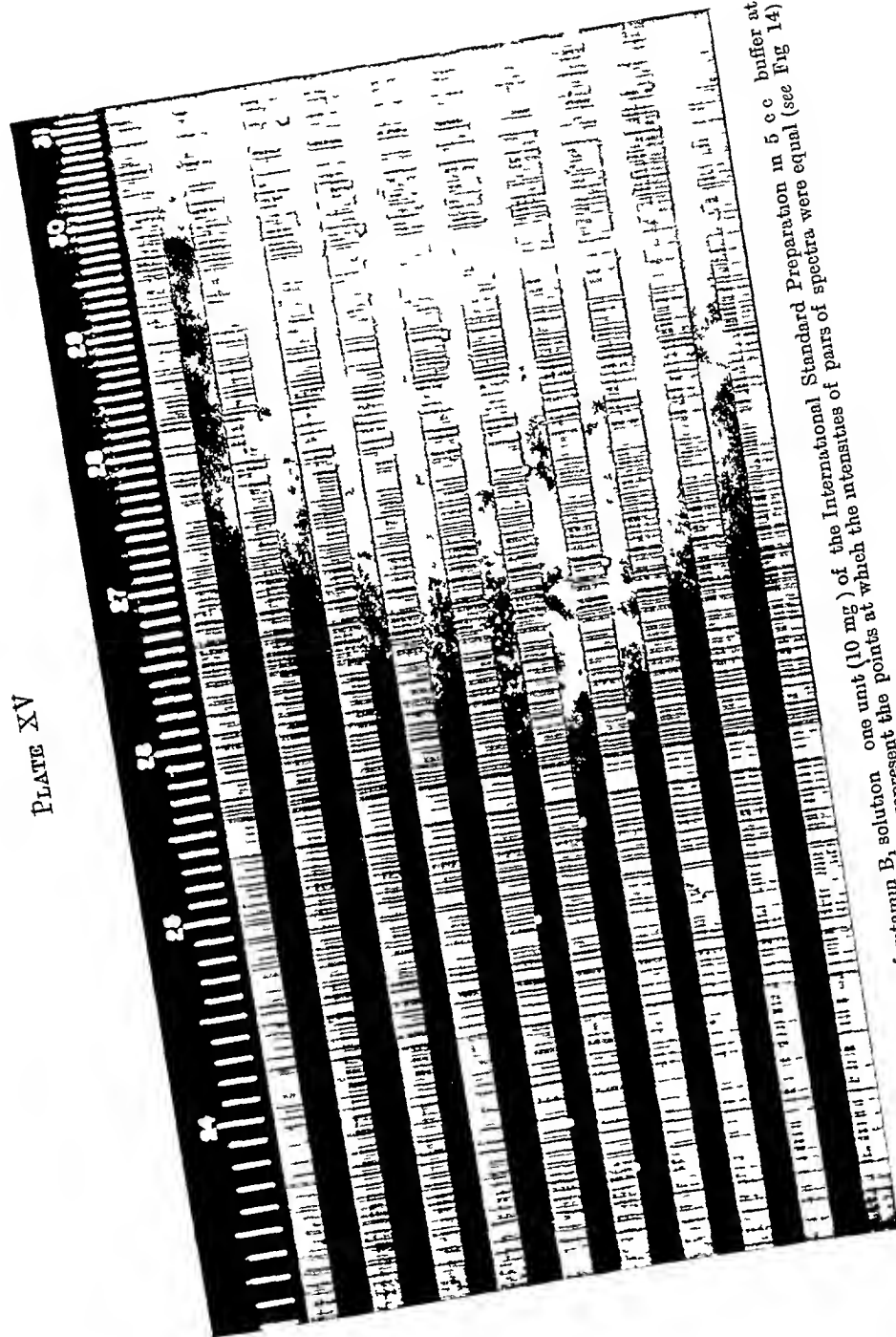
Absorption photograph of vitamin B_1 solution one unit (10 mg) of the International Standard Preparation in 5 c c buffer at
pH 7 Enlargement $\times$ 2 The white dots represent the points at which the intensities of pairs of spectra were equal (see Fig 14)
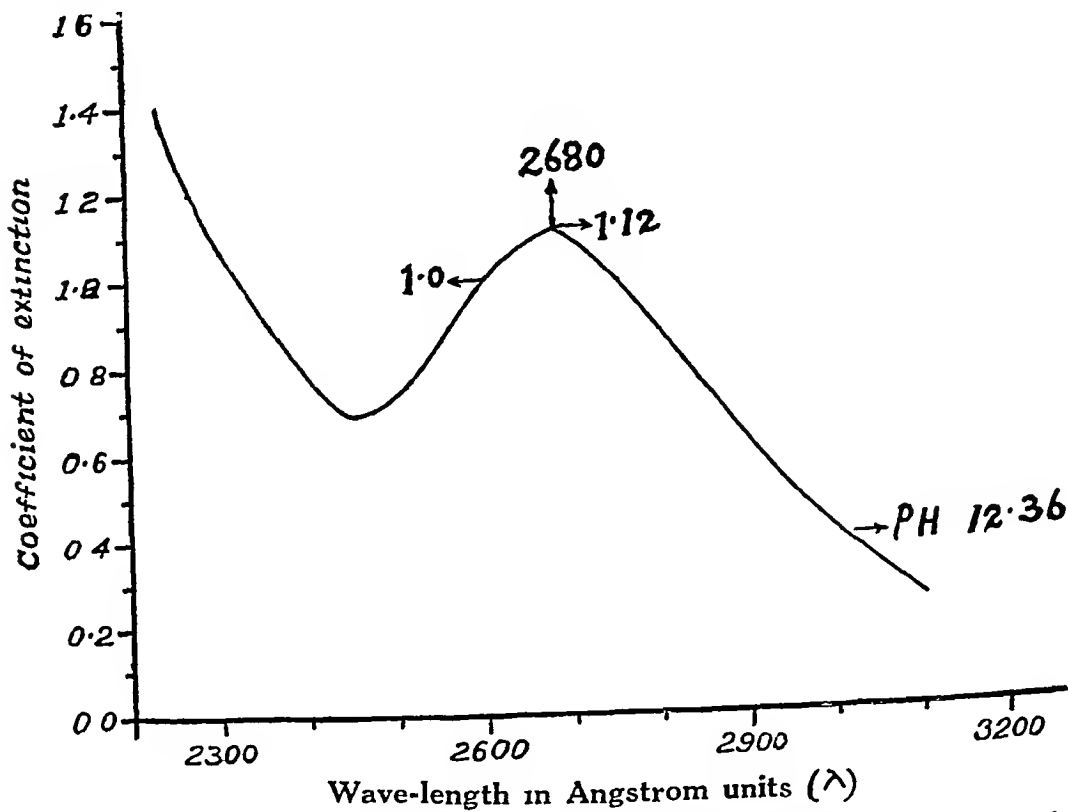
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Fig 11   Absorption curve of vitamin B_1 solution obtained from 64 8 mg International Standard
Preparation in 5 c c buffer at pH 1 2   Maximum absorption occurred at 2500 A U , extinction
coefficient for wave length 2600=0 56

Fig 12   Absorption curve of vitamin B_1 solution obtained from 122 2 mg International
Standard Preparation in 5 c c buffer at pH 2 97   Maximum absorption occurred at 2540
A U , extinction coefficient for wave length 2600=0 52

Studies on Vitamin B_1.

Fig 13   Absorption curve of vitamin B_1 solution obtained from 172 mg International Standard Preparation in 5 c c buffer at pH 3 53   Maximum absorption occurred at 2550 A U , extinction coefficient for wave length 2600=0 56
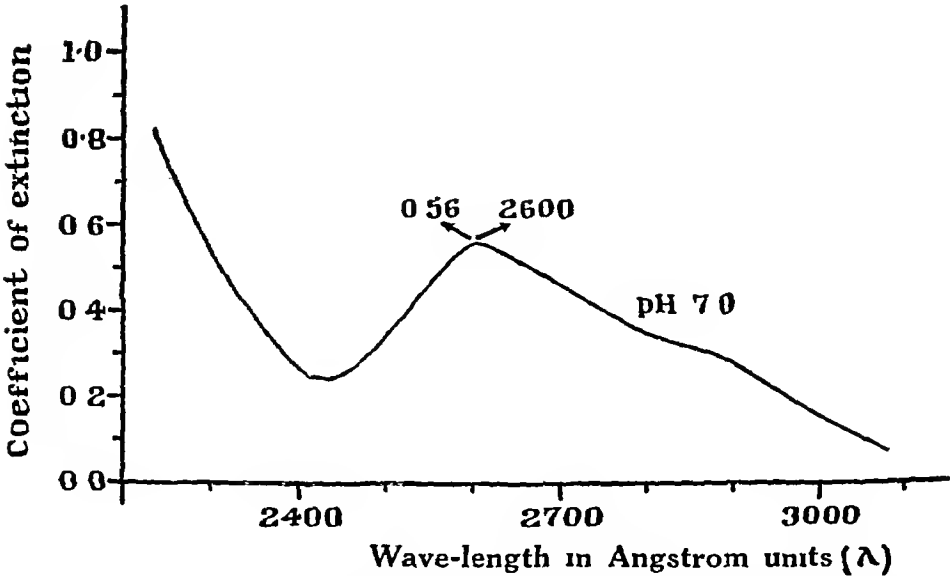
Fig 14   Absorption curve of vitamin B_1 solution obtained from 10 mg International Standard Preparation (i e , one unit) in 5 c c buffer at pH 7 0 Maximum absorption occurred at 2600 A U , extinction coefficient for wave length 2600 = 0 56
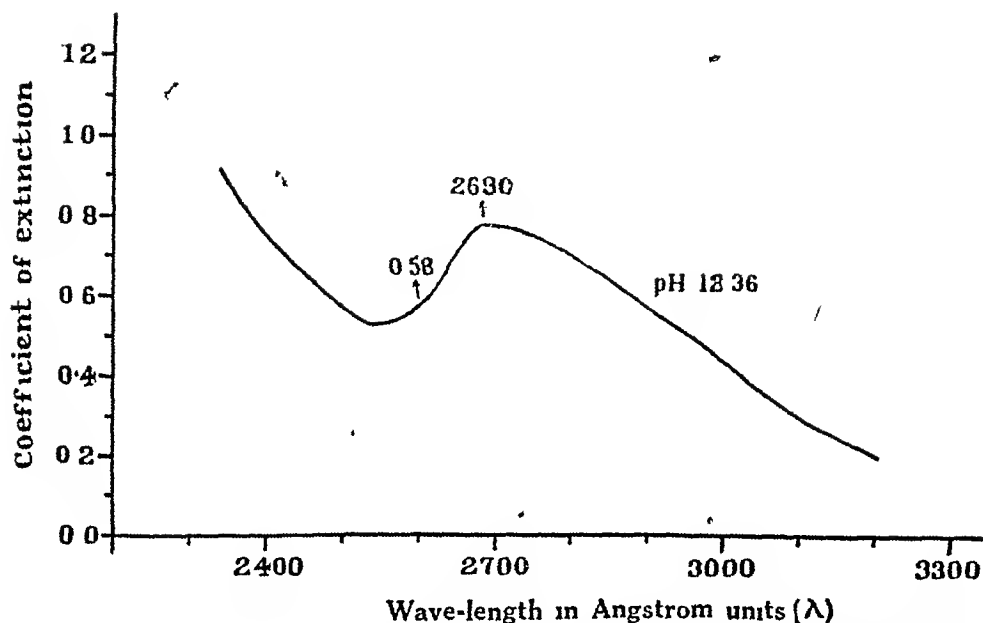
Fig 15   Absorption curvo of vitamin B₁ solution obtained from 5 5 mg International Standard Preparation in 5 c c buffer at pH 12 36   Maximum absorption occurred at 2680 A U , cstinction coefficient for wave length 2600—0 58

Summary

(1) A relation between the solubility of vitamin B₁ (from the International Standard Preparation) and pH has been found , solubility is at its minimum at about pH 3 5 , it increases slightly with increasing acidity and rapidly with increasing alkalinity

(2) The λ-maximum shifts with pH and the relation of the one to the other is represented by a straight line   with rising pH the shift is towards a longer wave-length, with falling pH it is towards a shorter

(3) The iso-electric point of vitamin B₁ seems to be about pH 3 5

(4) A method of obtaining standard solutions of vitamin B₁ from the International Standard Preparation is described

We desire to express our indebtedness to Sir Robert McCarrison for his help at every stage of this work

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J, MR

to accept the conclusion of Birch and Guha that the isoelectric point of vitamin B₁ is at a hydrogen-ion concentration above 8 5

Method of investigation

The method of investigation adopted by us was that of electrophoresis of a suspension of the International Standard Preparation of vitamin B₁ at levels of pH ranging from 1 04 to 13 07　Our objects were to determine the direction of migration of the vitamin at different levels of pH, the quantity migrating (or the velocity of migration) and the hydrogen-ion concentration at which the migration of the vitamin changed its direction (the isoelectric point)　In all our experiments we used a suspension—made up of 500 milligrams of the above Preparation in 5 c c buffer of the required pH, thus ensuring that, whatever the level of pH, vitamin B₁ was always in excess in the medium　Two means of achieving our objects were open to us　the so-called 'rising boundary method', and the spectrographic method　We have employed both, but whereas the former was of use for qualitative estimation, it was found to be impracticable for quantitative estimations Vitamin B₁, as present in the International Standard Preparation, is adsorbed on kaolin　On the subjection of a suspension of this adsorbate to electrophoresis a faintly turbid and slowly ascending stream was seen to pass up the side-arm of the positive cell of our apparatus　The turbidity was no doubt due to particles of kaolin on which the vitamin was adsorbed　But this phenomenon was visible to the unaided naked eye only on the alkaline side of pH 7, moreover, the upper boundary of the turbidity was not clearly defined and could not be accurately measured　On the acid side of pH 7 no such turbidity was apparent, yet, with the aid of a powerful beam of light and a reading lens, a Tyndal phenomenon could be observed in the apparently clear fluid in the side-arms of the electrophoretic cells This phenomenon was probably due to minute particles of kaolin, coated with the vitamin, streaming up the side-arm of the particular cell into which migration was occurring　Although it was impossible metrically to estimate the amount of migration yet this method of observation yielded interesting results (*vide infra*) We have relied, therefore, on the spectrograph for the full attainment of our objects The electrophoresis apparatus* we employed was that used in the work on the virus of Rabies (Sankaran *et al*, 1934*a*)　It was slightly modified to suit our present purpose　The modification consisted in the attachment of a short length of calibrated glass-tubing to the upper end of each cell (Text-figure), a glass-tap A being fitted to the top of each prolongation　These prolongations were marked as indicated in the figure　*a*, *b* and *c* on the negative cell and *a₁*, *b₁* and *c₁* on the positive cell, the gradings being identical in each　The object of these markings was to ensure that precisely the same amount of fluid could be withdrawn from each cell for spectrographic examination following electrophoresis of the suspensions

Prior to electrophoresis each cell is thoroughly cleansed and the agar gel seal B renewed　The cells are then filled with buffer of the required pH, the level of the buffer being accurately adjusted to the marks *a* and *a₁*　The side-arms C of the

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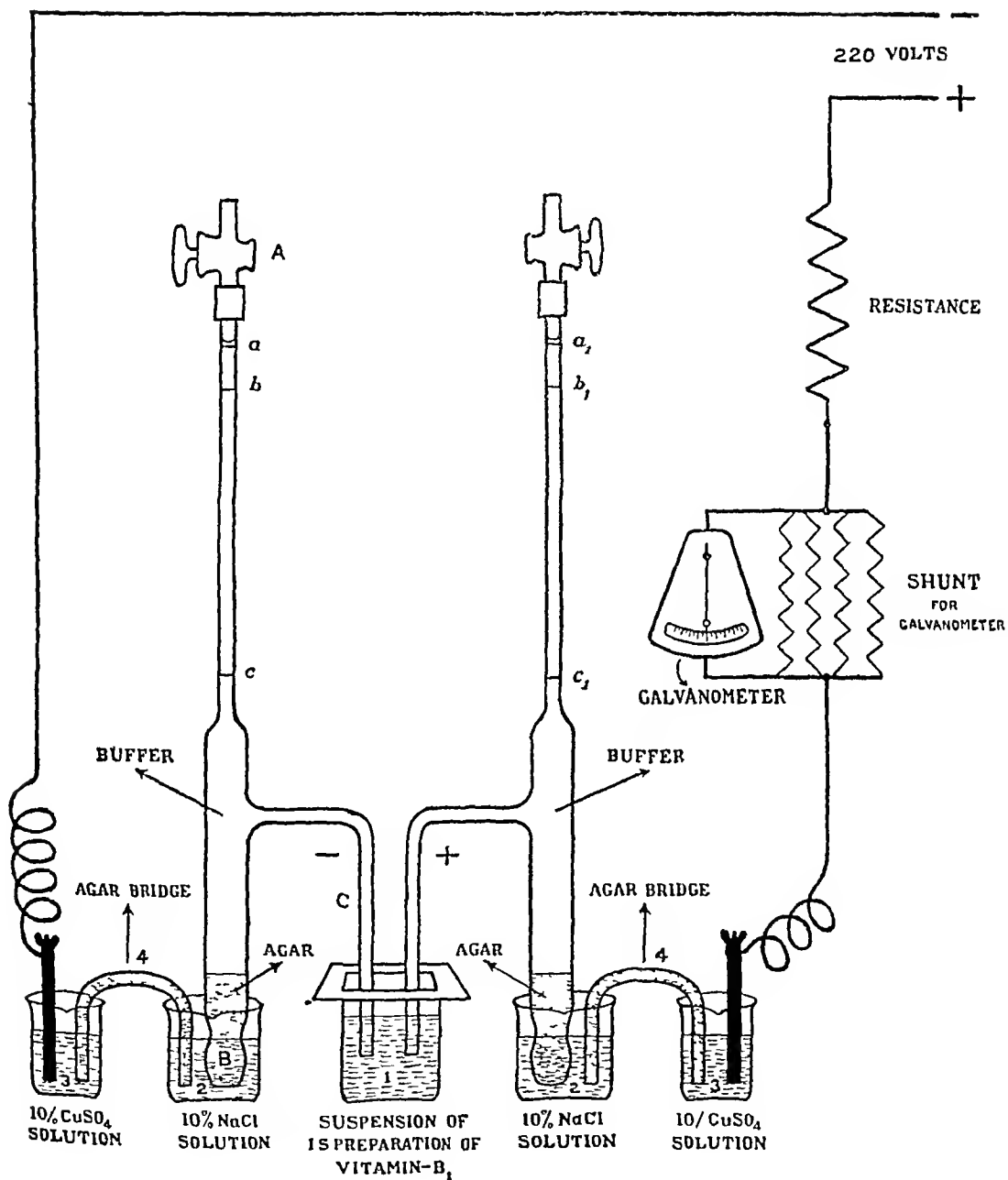
TEXT FIGURE

Showing electrophoresis apparatus and electrical connexions For descriptions see text

two cells are then immersed in the suspension, the suspension being made up in buffer of the same pH as that filling the cells. The current is then turned on and allowed to run for a fixed length of time. The same strength of current—two milliamps—was used in all our experiments. This was ensured by including in the circuit a variable resistance to give the same deflection of a galvanometer needle. The current gave rise to no generation of heat. The duration of its passage varied in different experiments from 45 minutes to $4\frac{1}{2}$ hours. The reason for this variation will presently appear. At the end of the required period the two cells are removed from the circuit, only after their removal is the current switched off. The side-arms of the cells are then carefully wiped with filter-paper, the taps at A are opened and the contents of the cells are run out until their level reaches the points at b and b_1. The fluid so run out is discarded. A measured quantity of the contents of both cells is then collected in appropriate vessels. This is done by allowing their contents to run out until the level reaches the point c or c_1 as the case may be. By this procedure equal amounts of material from the side-arms of the two cells are obtained for spectrographic study.

The amount of vitamin B_1 collected in this way in a fixed time is a measure of the velocity of migration, and accurate estimation of this amount gives a numerical value to this velocity. For this estimation we have relied on the property of vitamin B_1 to absorb ultra-violet light at A U 2600. The extinction coefficients at this wave-length and at wave-lengths of absorption maxima at different levels of pH are used as measures of the amount of vitamin that has migrated either to the cathode or to the anode. Full details of the spectrographic procedure are given in the preceding paper (Sankaran and De, *loc cit*).

In order to satisfy ourselves that reliance could be placed on the property of vitamin B_1 to absorb ultra-violet light at wave-length A U 2600, we adopted the following procedures :—

(a) A concentrate of the vitamin was prepared* from rice-polishings after the manner of Jansen and Donath (1927). Spectrographic examination of this concentrate in buffer of pH 7 0 and in normal NaOH solution gave absorption maxima at A U 2600 and 2730 respectively (Figs 1 and 2), thus agreeing well with our previous findings for the International Standard Preparation (Sankaran and De, *loc cit*)

(b) Vitamin B_1 is said to be destroyed by boiling in $N/2$ NaOH solution for one hour (Kinnersley and Peters, 1928), the biological test being used to determine its destruction. We, therefore, boiled the International Standard Preparation as well as the above-mentioned concentrate of vitamin B_1 in normal NaOH for one hour, which treatment, it may be assumed, destroyed all vitamin B_1. We then subjected both to spectrographic examination and found that both gave identical absorption maxima at A U 2810 (Figs 3 and 4). So that the substance absorbing light at this wave-length is not vitamin B_1, though it may be a product of its disintegration. The questions raised by this observation are interesting but outside the scope of the present paper

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* By Mr S Ranganathan of the Chemical Department of these Laboratories
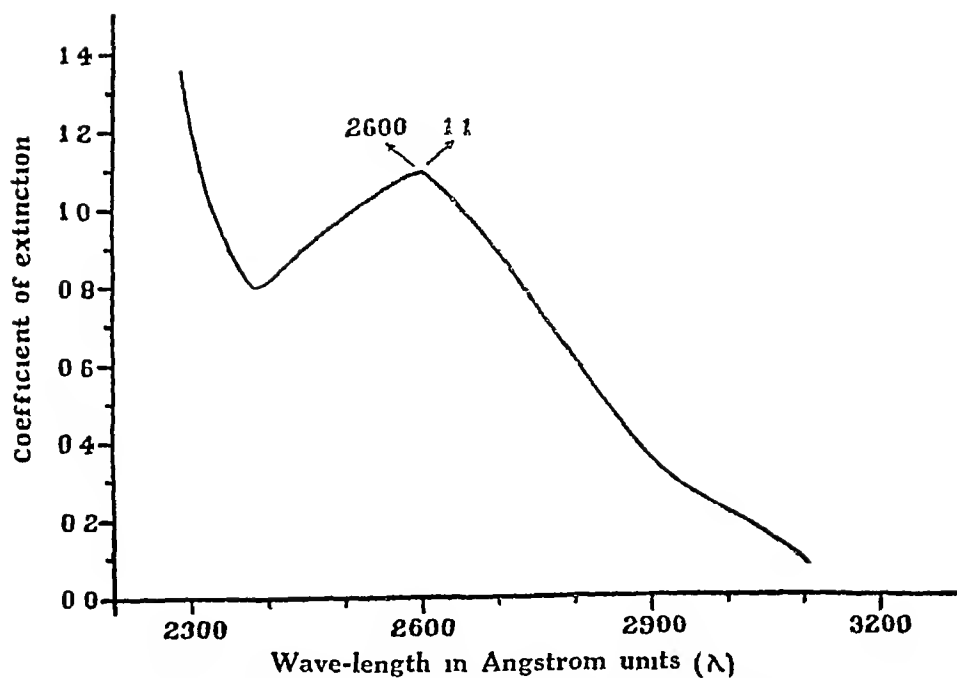
Fig 1   Absorption curve of vitamin B_1 concentrate prepared in this laboratory according to Jansen and Donath's method at pH 7 0
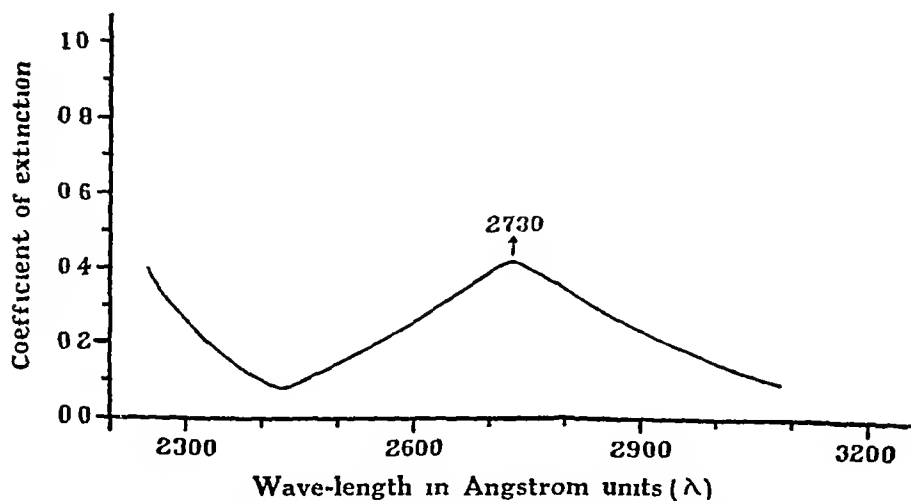
Fig 2   Absorption curve of the vitamin B_1 concentrate in normal NaOH

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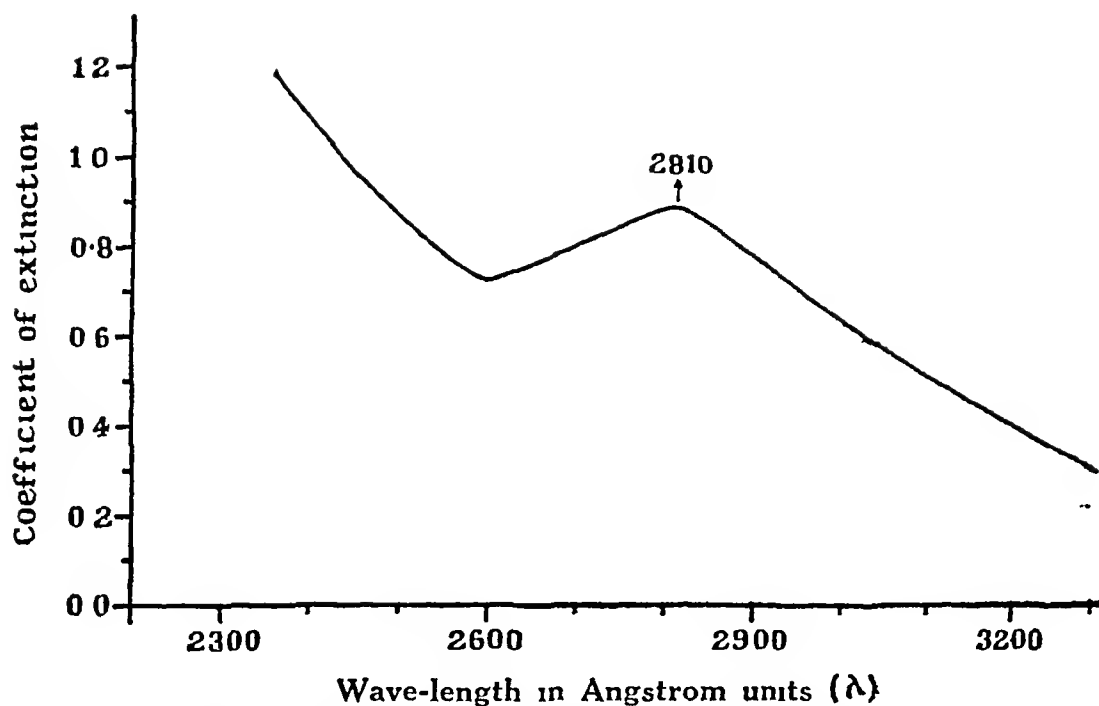
Fig 3   Absorption curve of the International Standard Preparation after boiling for one hour with normal NaOH
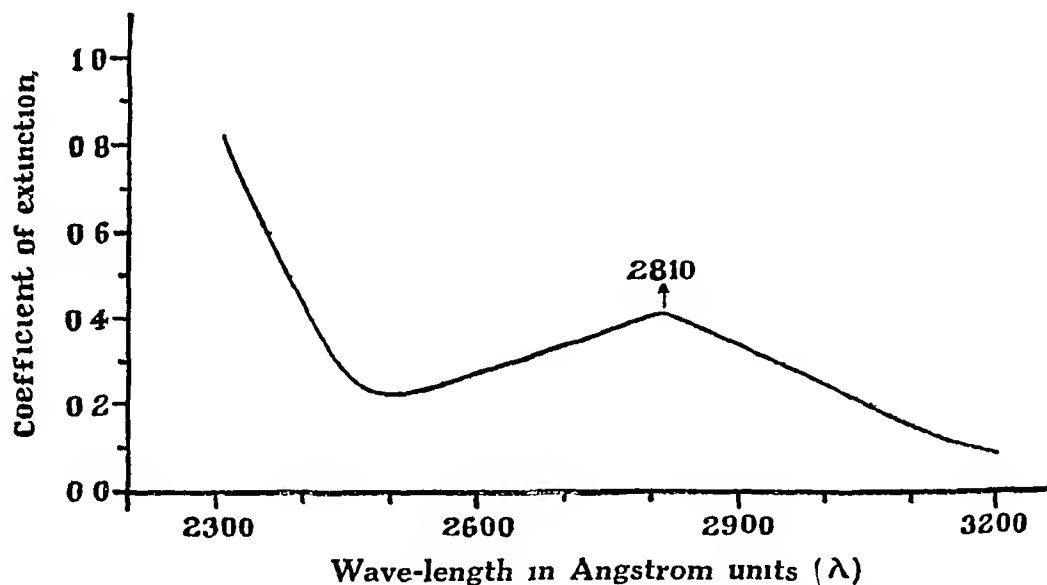
Fig 4   Absorption curve of the vitamin B₁ concentrate after boiling for one hour in normal NaOH

We conclude from the results of these procedures that the spectrographic method of estimating vitamin B_1 can be confidently relied upon, provided due count is taken of the shift in wave-length, consequent on change of pH, at which maximum absorption occurs (Sankaran and De, *loc cit*)

Electrophoresis experiments

We have performed the electrophoresis throughout the whole range of available buffers These buffers were made up according to the details given by Clark (1920) We were restricted to the use of borate, citrate and phosphates, since these do not absorb ultra-violet light Glycocol and phthalate could not be used as they completely absorb the light waves in the ultra-violet region Observations were made at 16 levels of pH, ranging from 1 04 to 13 07, thus practically covering the whole range of hydrogen-ion concentration that is of any significance in work of this nature It was necessary to carry out repeat experiments at certain levels of pH, so that we have in all made 24 observations

On the acid side of pH 7 electrophoresis was continued for over 4 hours since the migration velocities were very slow, and with shorter exposures to the electric current enough of the migrated vitamin could not be obtained to give satisfactory absorption curve maxima Failure to get a complete curve for quantitative purposes was one of the causes of repeat observations at acid levels of pH On the alkaline side of pH 7 we found that absorption was more and more complete with increasing pH, and here again repeat observations were necessary in order to secure complete absorption curves We adopted two methods to secure satisfactory curves at high levels of pH (*a*) electrophoresis for a shorter time—as short as 45 minutes at pH 13 07, and (*b*) dilution of the fluid obtained from the side-arm of the positive cell—as much as 27 times at pH 12 38 Either method gave satisfactory, and both methods gave like results All migration-values were calculated for a standard period of electrophoresis and for a standard volume of medium, namely, 4 hours and no dilution The resultant values, set out in columns 7 of Tables I and II, are comparable one with another inasmuch as the strength of the current was the same in all cases The experiments were carried out at the ordinary temperature of this Laboratory, this did not vary to any significant extent, the minimum being 18°C and the maximum 20°C

The results

These may be considered under two headings naked-eye observations and spectrographic observations

Naked-eye observations —Reference has already been made to these and to their limitations The migration of the vitamin in solution cannot, of course, be observed with the naked eye But when adsorbed on kaolin it is possible to observe the direction of migration, though not to estimate its quantity The ascending turbidity in the side-arm of the positive cell, when alkaline buffers are in use, makes such observation easy, and the Tyndal phenomenon in the apparently clear contents of the side-arms, when acid buffers are in use, is readily revealed by the beam of a Pointolite In all our experiments we have watched the direction of migration in these ways It was noted that from pH 4 45 to pH 13 07 migration was always towards the positive pole, from pH 1 04 to pH 1 42 it was always towards the negative pole From pH 1 92 to pH 3 95 migration towards both poles was sometimes, though not always, observed

Spectrographic observations —In all our experiments material collected in the way described, both from the side-arm of the positive cell and from that of the

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negative cell, was subjected to spectrographic examination   The results of the experiments, wherein we succeeded in obtaining satisfactory absorption curves, are set out in Tables I and II

TABLE I

Giving the results of spectrographic examination of material derived from the positive and the negative cells of the apparatus following electrophoresis of suspensions of 500 mg I S Preparation of vitamin B_1 in 5 c c buffer of varying pH

Number of observations	pH	Period of electrophoresis, hours	DILUTION OF MATERIAL DERIVED FROM		OBSERVED EXTINCTION COEFFICIENTS FOR WAVE LENGTH 2600 A U GIVEN BY DILUTED MATERIAL FROM		CALCULATED EXTINCTION COEFFICIENTS FOR 2600 A U UNDER STANDARD CONDITIONS (NO DILUTION AND 4 HOURS ELECTRO PHORESIS) MATERIAL FROM	
			Positive cell	Negative cell	Positive cell	Negative cell	Positive cell	Negative cell
(1)	(2)	(3)	(4)	(5)	(6)		(7)	
1	1 04	4	×2 5	×2 5	0 0	0 42	0 0	1 05
2	1 42	4	×2 0	×3 0	0 0	0 32	0 0	0 96
3	1 93	4	×1 4	×1 4	0 50	0 60	0 70	0 84
4	2 27	4	×1 0	×2 0	0 30	0 36	0 30	0 72
5	2 97	4	×2 0	×2 0	0 0	0 0	0 0	0 0
6	3 36	4	×1 5	×1 5	0 20	0 25	0 30	0 375
7	3 95	4	×1 3	×1 3	0 70	0 20	0 91	0 26
8	4 45	4	×1 5	×1 5	0 68	0 0	1 02	0 0
9	4 96	4½	×5 0	×5 0	0 48	0 0	2 13	0 0
10	5 97	3½	×5 0	×5 0	0 90	0 0	5 14	0 0
11	6 98	4	×6 0	×2 0	1 00	0 0	6 0	0 0
12	8 04	3	×6 0	×2 0	0 98	0 0	7 84	0 0
13	9 24	1	×2 5	×2 0	0 90	0 0	9 0	0 0
14	9 97	1½	×6 0	×3 0	0 70	0 0	11 20	0 0
15	12 38	1	×27 0	×2 0	0 44	0 0	47 52	0 0
16	13 07	?	×18 0	×2 0	0 76	0 0	72 96	0 0

TABLE II

Giving the results of spectrographic examination of material derived from the positive and the negative cells of the apparatus following electrophoresis of suspensions of 500 mg I S Preparation of vitamin B_1 in 5 c c buffer of varying pH

Number of observations	pH	Period of electro phoresis, hours	Dilution of material derived from		Observed wave length of maximum absorption	Observed extinction coefficients at wave length of maximum absorption given by material from		Calculated extinction coefficients for wave length of maximum absorption under standard conditions (no dilution and 4 hours electrophorfsis) Material from	
			Positive cell	Negative cell		Positive cell	Negative cell	Positive cell	Negative cell
(1)	(2)	(3)	(4)		(5)	(6)		(7)	
1	1 04	4	×2 5	×2 5	2500	0 0	0 52	0 0	1 30
2	1 42	4	×2 0	×3 0	2510	0 0	0 36	0 0	1 08
3	1 93	4	×1 4	×1 4	2510	0 35	0 72	0 77	1 008
4	2 27	4	×1 0	×2 0	2540	0 40	0 42	0 40	0 84
5	2 97	4	×2 0	×2 0		0 0	0 0	0 0	0 0
6	3 36	4	×1 5	×1 5	2545	0 30	0 31	0 45	0 465
7	3 95	4	×1 3	×1 3	2550	0 74	0 25	0 962	0 325
8	4 45	4	×1 5	×1 5	2580	0 69	0 0	1 035	0 0
9	4 96	4½	×5 0	×5 0	2590	0 50	0 0	2 22	0 0
10	5 97	3½	×5 0	×5 0	2590	0 90	0 0	5 143	0 0
11	6 98	4	×6 0	×2 0	2600	1 00	0 0	6 0	0 0
12	8 04	3	×6 0	×2 0	2625	1 00	0 0	8 0	0 0
13	9 24	1	×2 5	×2 0	2650	1 18	0 0	11 8	0 0
14	9 97	1½	×6 0	×3 0	2660	0 84	0 0	13 44	0 0
15	12 38	1	×27 0	×2 0	2680	0 63	0 0	68 04	0 0
16	13 07	¾	×18 0	×2 0	2700	0 88	0 0	84 48	0 0

Columns 7 of Tables I and II give the calculated extinction coefficients for A U 2600 and for wave-lengths of maximum absorption*, corresponding to the absorption that would be given by the undiluted contents of the respective cells after four hours electrophoresis That is to say, they represent the quantity of vitamin B_1 that would migrate in the given time towards either anode or cathode Diagrams of these coefficients in relation to pH are given in Figs 5 and 6 It will be noted that at pH 2 97 no migration occurred either towards the positive or towards the negative pole , that below this level of pH and down to pH 1 93 migration occurred towards both poles , that above this level of pH and up to pH 3 95 bipolar migration also occurred , that below pH 1 93 migration was always towards the

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* For reasons stated in the preceding paper the extinction coefficients have been calculated both for wave length 2600 and for wave lengths of maximum absorption

negative pole, and that above pH 3 95 it was always towards the positive pole, its velocity increasing with increasing alkalinity   At the range of pH between 1 93
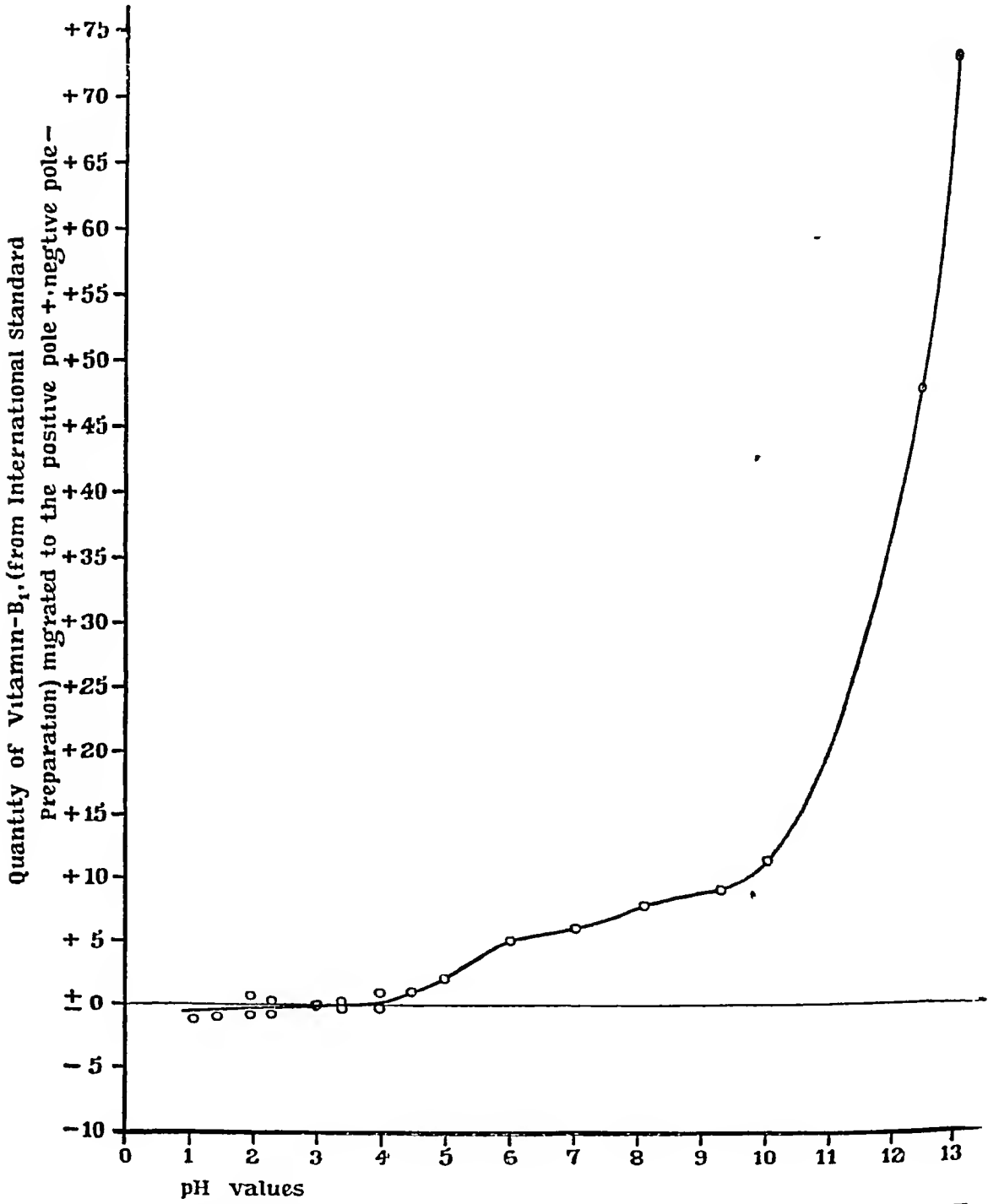
Fig 5   Showing migration of vitamin B_1 (International Standard Preparation) at levels of pH from 1 04 to 13 07 (*see* Table I), extinction coefficients calculated at A U 2600
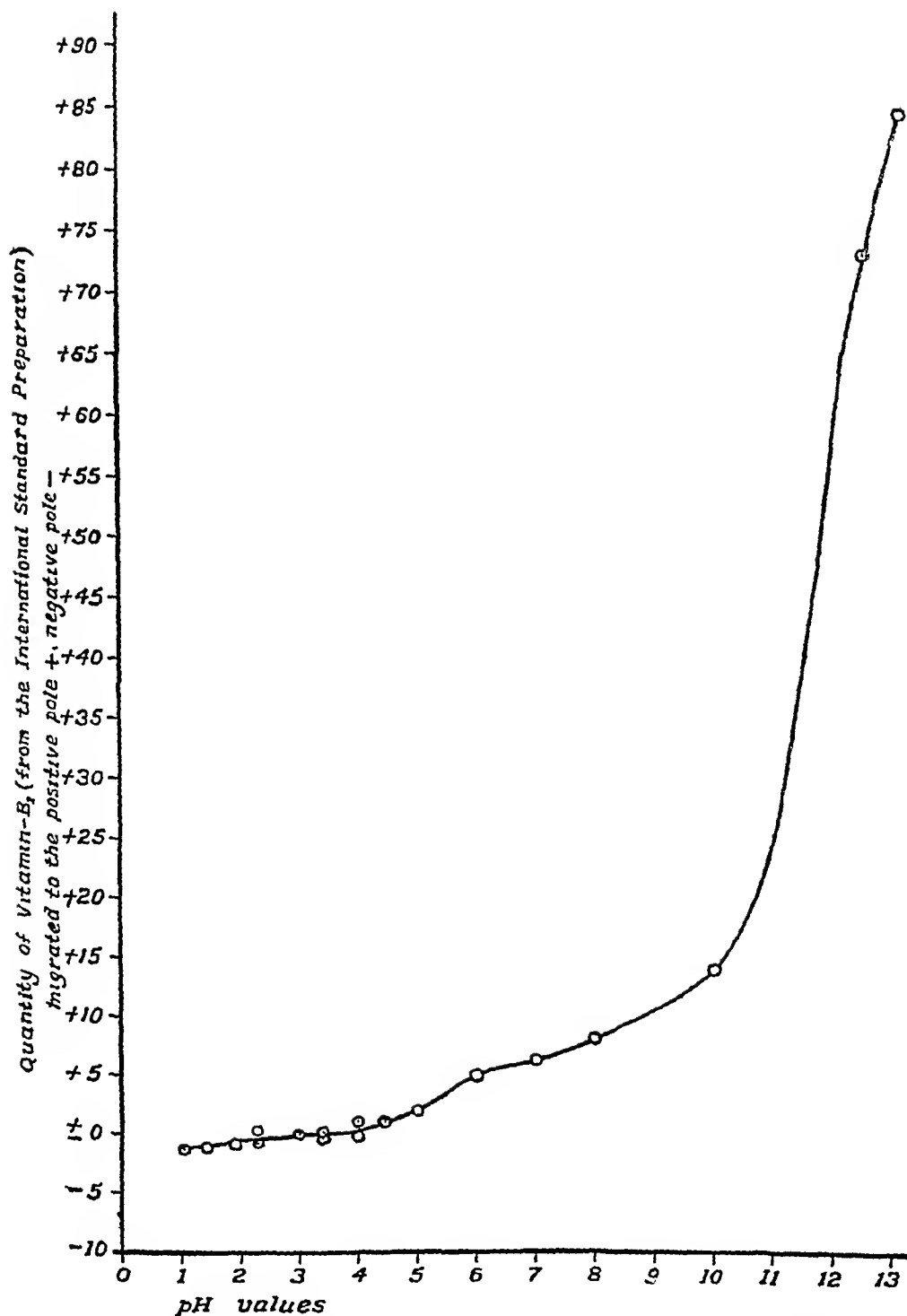
Fig 6   Showing migration of vitamin B₁ (International Standard Preparation) at levels of pH from 1 04 to 13 07 (see Table II), extinction coefficients calculated for wave lengths at which maximum absorption occurred   Note similarity between Figs 5 and 6

and 3 95 we have been at pains to confirm this phenomenon of bipolar migration by observations made at intervals of 0 5 pH, and to verify them by repeat experiments
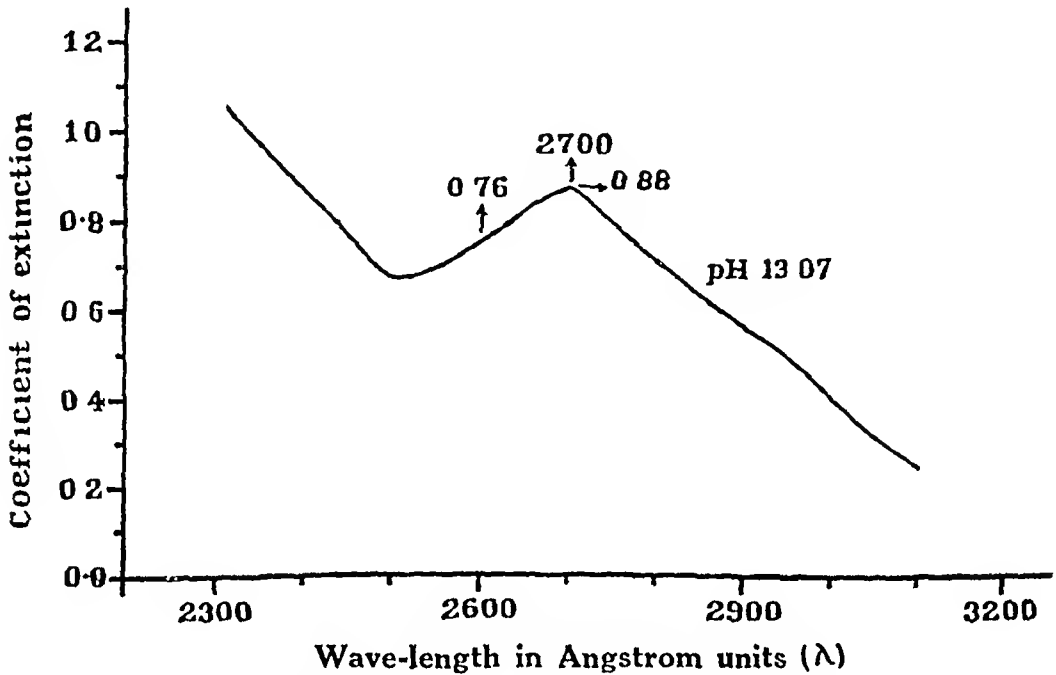
Fig 7   Absorption curve given by material from positive cell at pH 13 07   Migration occurred towards positive pole only
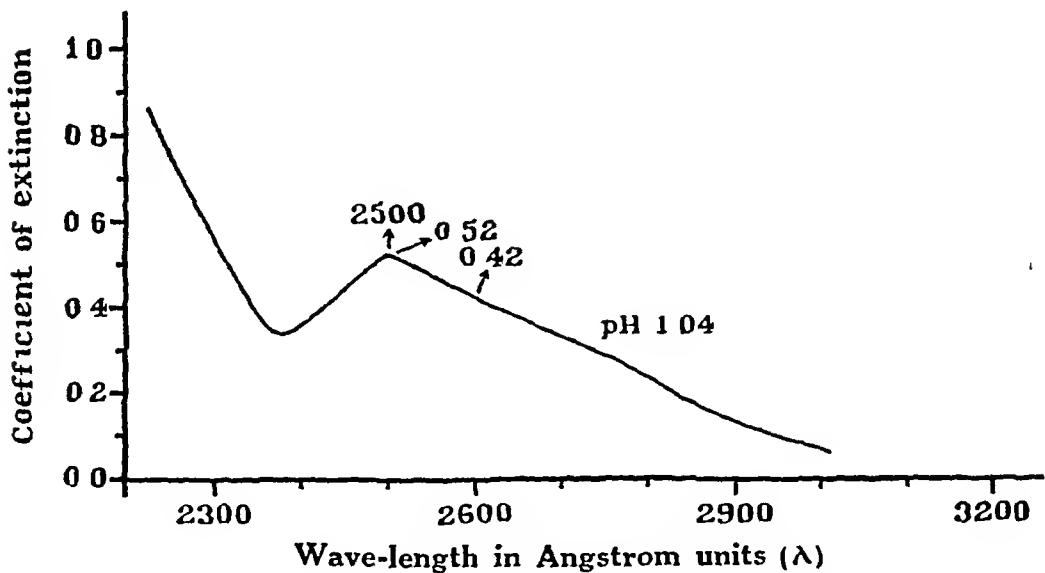
Fig 8   Absorption curve given by material from the negative cell at pH 1 04   Migration occurred towards negative pole only

Figs 7 and 8 are representative curves of absorption yielded by buffer wherein migration had occurred towards the positive and negative poles respectively

PLATE XVI
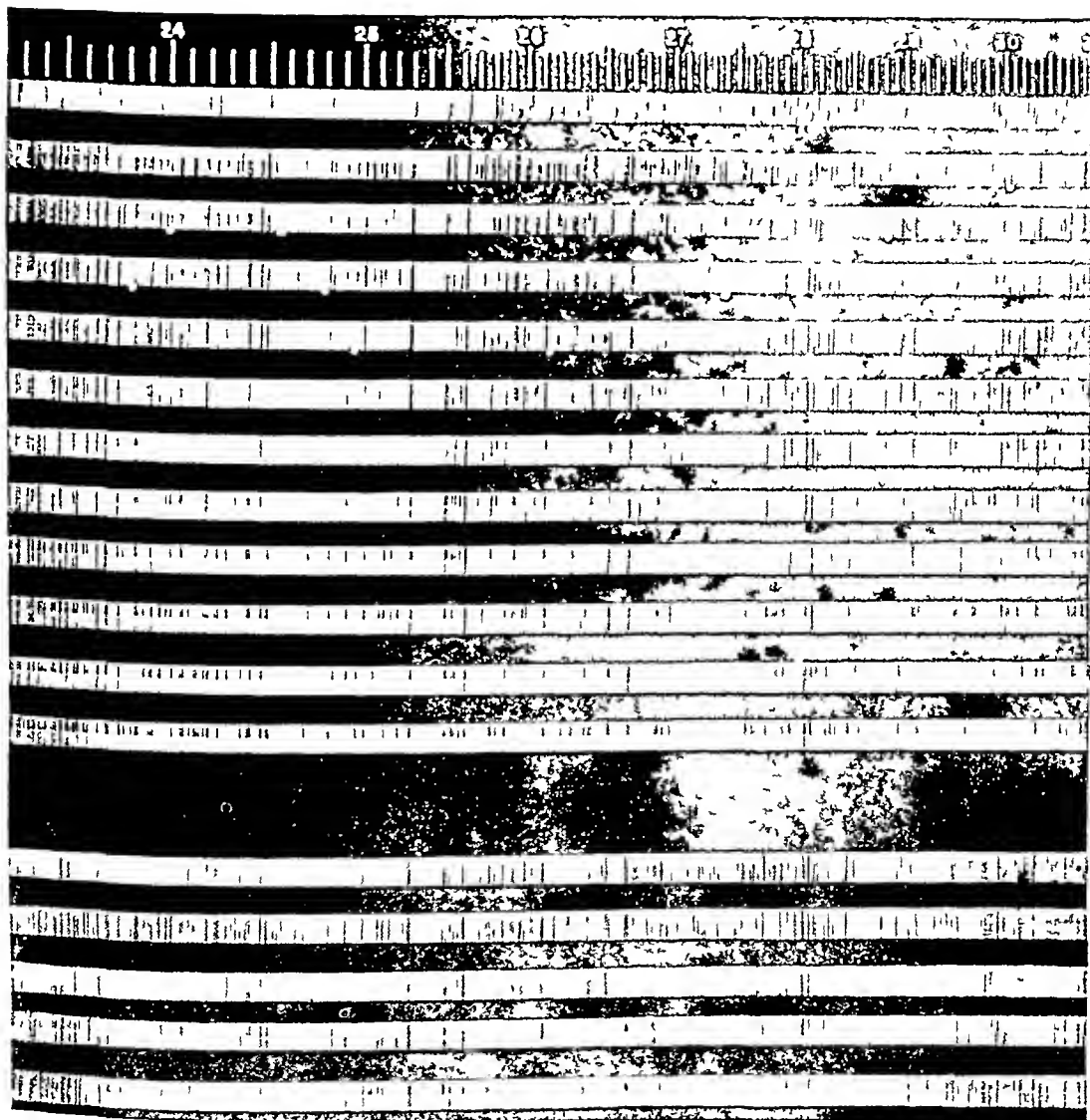
Spectrophotograph (×2) of material obtained from both cells at pH 1 42   Absorption occurred in material from negative pole only
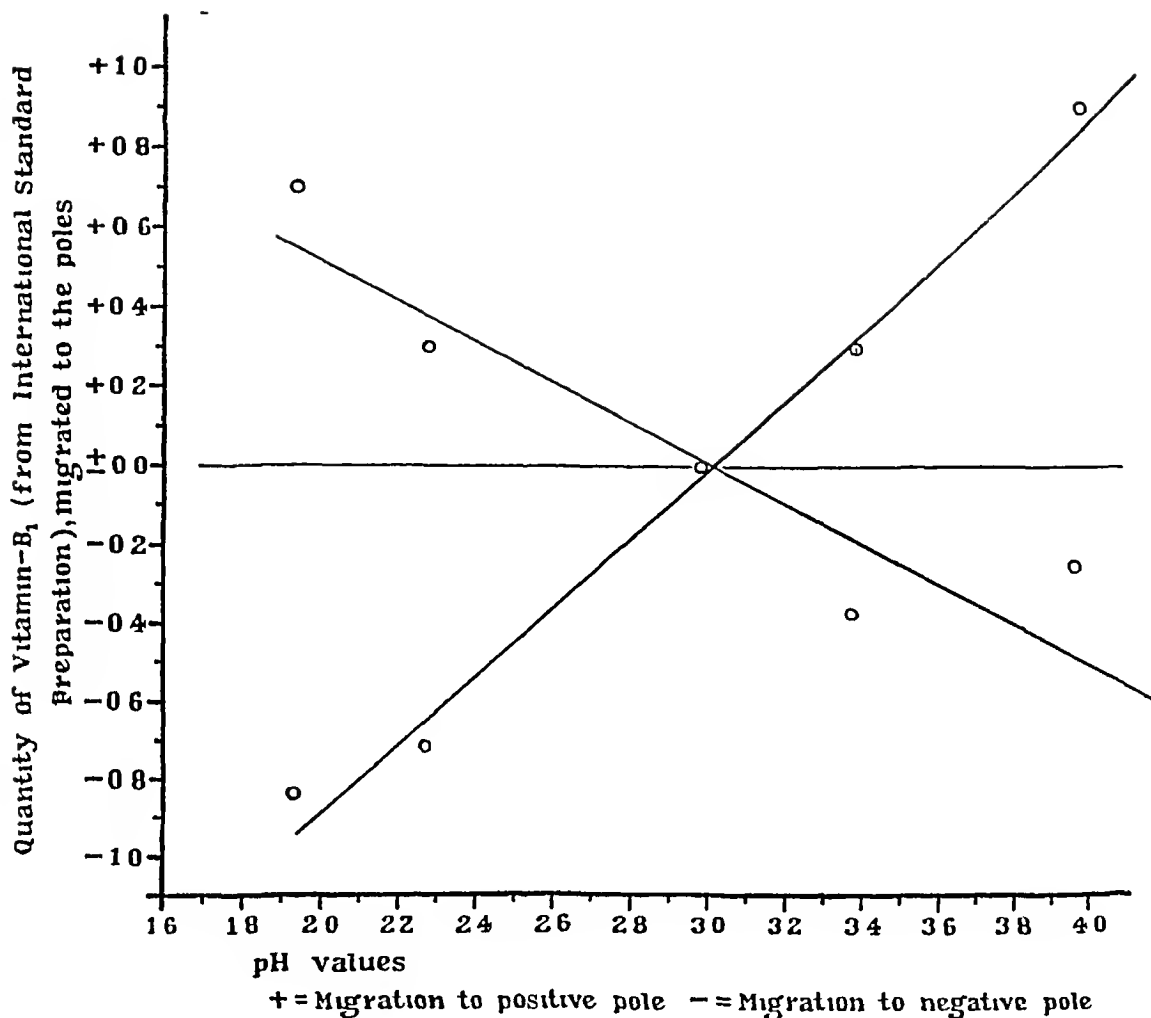
Fig 11 Showing migration of vitamin B$_1$ (International Standard Preparation) at pH levels from 1 93 to 3 95 Lines of best fit joining extremes of bipolar migration intersect at about pH 3 on line of zero velocity (*see* Table I)

Conclusion

The results here recorded show clearly that vitamin B$_1$ behaves as an amphoteric electrolyte Regarding the actual value of the isoelectric point the bipolar migration over a range of 2 0 pH-units makes the problem perplexing , for it introduces the possibility that vitamin B$_1$, as contained in the International Standard Preparation, is not a pure substance but a complex of substances Similar bipolar migration has been observed by Geiger (1931) when working with oxyhæmoglobin , while Howitt and Prideaux (1932) refer to Reiner (1927)* as having spoken of a broad ' isoelectric zone ' in connexion with serum globulin According to these observers the substances with which they were dealing were not

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* Reference not available in Coonoor

THE IODINE-CONTENT OF INDIAN FOOD-STUFFS

BY

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WITHIN recent years much work has been done on the iodine-content of various food-stuffs notably by Fellenberg in Switzerland, by Hercus and his co-workers in New Zealand and by McClendon in the United States of America From the data thus provided Orr and Leitch (1929) have concluded that the minimum quantity of iodine required for equilibrium by an adult male is 15γ per day and by a child about 50γ , but to provide a fair margin of safety, for such factors as exercise and excitement, an adult male is stated to require 45γ and a child 150γ of iodine per day Josephus Jitta (1933) considers that a daily intake of 120γ of iodine is just sufficient to prevent the appearance of goitre in school children According to Cameron (1932) a man's requirement is at least 35γ to 70γ of iodine per day He further states that ' in many parts of the world diets may not contain even this minute trace, in which case a large proportion of children and young adolescents develop simple goitre ' McClendon (1933), as a result of his study of the world distribution of goitre, considers that Japan is the only non-goitrous country , the incidence being as low as one per million He states that as sea-weeds, containing at least 1,000 times as much iodine as any other foods, are constant constituents of the diet of the Japanese, their average daily intake of iodine is easily double that of any other people In view of these observations and of the conclusions that have been drawn from them it was thought desirable to determine the amounts of iodine contained in some of the commoner food-stuffs of India

Technique

Iodine was estimated by the improved micro-method described in a previous paper (Patnaik, 1933) This method involves the use of pure K MnO$_4$ But even the purest samples of the salt, as received in bottles from the manufacturer, invariably contain traces of iodine amounting in some samples to as much as 6γ per kg To get rid of this iodine it has been found necessary to recrystallize the permanganate The method previously adopted of estimating iodine in K MnO$_4$ was as follows —

Fifty grammes of K MnO$_4$, after reduction with ethyl alcohol in alkaline medium, were filtered quantitatively through a Buchner funnel The clear filtrate was

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evaporated to dryness in five nickel dishes after the addition to each dish of 3 g of K_2CO_3 and 0 2 g of cane-sugar After incinerating this residue, extraction of the iodide with alcohol and colorimetric estimation of iodine were carried out as usual In this method the amount of ash left in the dishes after incineration was found to be considerable , because, on the whole, as much as 15 g of K_2CO_3 were added to prevent melting of the mass during incineration If only very little K_2CO_3 was added instead of 15 g the mass became fluid and would seldom set well for alcoholic extraction The increase in the bulk of the ash, consequent on the use of greater quantities of K_2CO_3 was, therefore, inevitable To avoid such an increase in the bulk the method used in the present investigation was modified as detailed below —

One hundred grammes of pure K MnO_4 were dissolved in a litre of hot distilled water contained in a porcelain evaporating basin and 10 c c of a ten per cent solution of KOH were added The permanganate was then reduced by the gradual addition of 20 g to 22 g of cane-sugar Thereafter the solution was filtered through a Buchner funnel The residue was washed several times with distilled water and finally it was washed thrice with absolute alcohol , 5 c c being used at each washing The clear filtrate was then evaporated with 2 g of K_2CO_3 to about 50 c c over an evaporating basin This solution was distributed between two nickel dishes, evaporated to dryness and slowly ignited at below red-heat until the whole of the organic smell disappeared During incineration, the mass, unlike that in the previous method, easily yielded a smooth powder without any further addition of K_2CO_3 The paste got out of this powder was found to set as well for alcoholic extraction as in the case of food-stuffs The rest of the processes of extraction, clarification and estimation remained the same

In all other respects the method of iodine-determination in food-stuffs was that previously described (Patnaik, *loc cit*) This method has, in my hands, proved to be more accurate and more convenient than others, especially when relatively large charges of the materials to be estimated are used It is obviously well to use as large charges as possible so as to secure the highest yield of iodine for colorimetric estimation

A list of 100 Indian food-stuffs whose iodine-content was determined by the above means is given below The vernacular names [Hindi (H), Bengali (B), Tamil (Tm), Telugu (T), Malayalam (M), Canarese (C) and Oriya (O)] of these food-stuffs are given in addition to the English and botanical names

Results.

The results of these analyses show that iodine is present in all the Indian food-stuffs but that the amount varies not only in different food-stuffs but in different samples of the same food-stuff In general cereals contain from 18γ to 65γ of iodine per kg , millets from $12 4\gamma$ to 101γ, pulses from $22 5\gamma$ to $33 8\gamma$, oil-seeds and nuts from $6 9\gamma$ to $45 1\gamma$, root vegetables from $4 5\gamma$ to $17 5\gamma$, garden produce and green vegetables from $4 5\gamma$ to $77 7\gamma$, condiments and spices from $zero\gamma$ to 507γ , dairy, fishery and farm products from 18γ to $5,505\gamma$, and, narcotics, intoxicants and other miscellaneous products from $zero\gamma$ to 169γ

TABLE I

Giving the iodine-content of 100 Indian food-stuffs

Group	Serial number	Sample	Source	Iodine content per kg
I. Cereals	1	Raw rice (washed) *Oryza sativa*, H Kancha chaul, B Atap chaul, Tm Pachu arisi, T Pachi beemu, M Pachæ ari, C Hasalukki, O Orua chaulo	Nellore	20 3γ
	2	Raw rice (unwashed)	,,	31 6γ
	3	Parboiled rice (washed), H Ubelhuvre chaul, B Sidho chaul, Tm Pulungal arisi T Uppudu beemu, M Punngal ari, C Kusalukki, O Usuna chaulo	,,	18 1γ
	4	Parboiled rice (unwashed)	,,	22 6γ
	5	Wheat, *Triticum vulgare*, H Giloom, B Gom, Tm Godumye T Godumalu, M Kotampam, C Godi, O Gohomo	Nilgiris	22 8γ
	6	Wheat flour, H Atta, B Anta Tm Godumanav, T Godnma pindi, M Godumbamava, C Goduvæ hudi, O Gohomochuna	Bombay	27 0γ
	7	American white flour, H Maida, B Moida, Tm Merikinmav, T Maidapindi, M Menkamava, C Markin, O Moida chuna	America	05 4γ
	8	Tinned oats, *Avena sativa*, H Javi	Market sample	22 6γ
	9	Barley, *Hordeum vulgare* H Jon	,,	18 0γ
II Millets	10	Sorghum, *Andropogan sorghum* H Juari, B Juar, Tm Cholam, T Jonnalu, M Cholam, C Jola, O Jonnা	Coimbatore District	29 3γ
	11	Ragi, *Eleusine coracana* H Mudder, B Mudor, Tm Kel vargu, T Chodulu, M Muttari, C Ragi, O Man lu	,,	101 4γ
	12	Bulrush or spiked millet, *Pennisetum typhoideum*, H Bajra, B Bojra, Tm Kamboo, T Gantelu, M Kamjam, C Sajje, O Ghantia	,,	45 1γ

Table I—*contd*

Group	Serial number	Sample	Source	Iodine content per kg
II Millets—*concld*	13	Italian millet, *Setaria italica*, H Kanguni, B Amandhan, Tm Tenye, T Korralu, M Tena, C Navana, O Kangoo	Combatore District	27 0γ
	14	Samai, *Panicum miliare*, H Savan, Tm Samai T Samulu, M Shama, C Samaye, O Snua	Nilgiris	38 4γ
	15	Maize (fresh), *Zea mays*, H and B Bhutta, Tm and M Makka cholam, T Mokka jonnalu, C Mekke jola, O Moka	,,	12 4γ
III Pulses	16	Red gram or pigeon pea, *Cajanus indica*, H Arhar, B Arhad, Tm Tuvarye, T Kandulu, M Tuvern, C Togari, O Horodo or Kandulo	Combatore District	22 5γ
	17	Green gram, *Phaseolus radiatus*, H Mung, B Mug, Tm Pachaipayer, T Pachapesalu, M Cherupayare, C Hesaru, O Mugo	Malabar	33 8γ
	18	Black gram, *Phaseolus mungolinn*, H Mash, B Kalai, Tm Voolundhu, T Minumulu, M Ulhundu, C Uddu, O Biri	,,	33 8γ
	19	Bengalgram, *Cicer arietinum*, H Chola, B Boot, Tm Kadalye, T Sanagalu, M Kadalakka, C Kadale, O Gujchonn or Buto	,,	22 5γ
	20	Horse gram, *Dolichos biflorus*, H Kooth, B Chhola, Tm Kolloc, T Ulavalu, M Muthra, C Huruli, O Kulutho	Combatore District	22 5γ
	21	Pea, *Pisum sativum*, H Puttham, B Chana, Tm Patahani, T and C Battum, M Payar, O Potthani chona	Nilgiris	22 5γ
IV. Oil-seeds and nuts	22	Gingelly, *Sesamum indicum*, H and B Til, Tm Ellu, T Nuvulu, M Ellu, C Yellu, O Rasi	Malabar	23 3γ
	23	Ground nut, *Arachis hypogea*, H Mungphali, B Chinabadam, Tm Nilakadalve or Verkadalye, T Vershanugalu, M Nilakadala, C Nilakadala, O Cherochona	Combatore District	6 9γ

	No.		Place	γ
	24	Mustard, *Brassica juncea*, H Sarshoom, B Shorsha, Tm Kadugu, T Sarasavalu or Avalu, M Katulu, C Sasivay, O Sorisho	Malabar	45 1γ
	25	Coco nut (edible portion), *Cocos rucifera*, H Narial, B Narikel, Tm Thengaui, T Kobburikaya, M Tenga, C Tengnakai, O Nodia	,,	18 1γ
	26	Coco nut water	,,	13 5γ *(per litre)*
V Tubers	27	Potato, *Solanum tuberosum*, H Alu, B Alu, Tm Urulai kilhangu, T Bangalidumpa, M Urlakilhangu, C Hanlu gadda, O Bilathi alu	Nilgiris	11 3γ
	28	Sweet Potato, *Ipomea batatus*, H and B Mitaha alu, Tm Surkarye vellikilhangu T Sotdumpa, M Chalkarni kil hangu, C Genusu, O Kondomools	Palghat	4 5γ
	29	Arrowroot, *Curcuma angustifolia* H Aruta, B Arorut Tm Araruttu, T Palagundi, M Kuvva, C Kuvegida, O Palu	Market sample	17 5γ
	30	Colocasia, *Colocasia antiquorum*, H Arbi B Kochu, Tm Chemang kilhangu, T Shamagada, M Chompakilhangu, C Chamagada O Siru	Palghat	4 5γ
	31	Carrot, *Daucus carota*	Nilgiris	100γ
VI Garden produce and green vegetables	32	Tamarind, *Tamarindus indica*, H Imli, B Tentool, Tm and M Puli, T Chintapandu, C Huli, O Thentuli	Malabar	11 3γ
	33(a)	Lemon (juice), *Citron decumana* H Nimboo, B Lebu, Tm Elimichum kau, T Nimmakaya, M Cherunaranga, C Nimbu hannu, C Lembu	,,	5 1γ *(per litre)*
	33(b)	Lemon peel	,,	nil
	34	Orange (juice) *Citron medicaxaracuda*, H Santara, B Kamla lebu, Tm Orengi T Kamalapandu, M Madranaranga, C Kithhal-rehanu, O Komola	Burliar	6 0γ *(per litre)*
	35	Mango (raw), *Mangofera indica* H Kachr aam, B Kanchaam, Tm Mangaui, T Maudikaya, M Mangr, C Mavinakai, O Koncha ambo	Malabar	9 0γ

TABLE I—*contd*

Group	Serial number	Sample	Source	Iodine content per kg
VI Garden produce and green vegetables —contd	36	Mango (ripe), H and B Pakka aam, Tm Mambalam, T Maudi pandu, M Manga palam, C Marinapalam, O Pachola ambo	Malabar	16·4γ
	37	Brinjal, *Solanum melogna*, H Bengan, B Begoon, Tm Kathari kaui, T Vonkaya, M Valluthunga, C Badinakai, O Bugono	Coimbatore District	6·8γ
	38	Tomato (raw), *Lycopersicum esculentum*, H Tomato, B Toch begoon, Tm Thakkalikaui, T Seemavonkaya, M Thakali kai, C Thakkali, O Bilathi bnigono	"	5·7γ
	39	Tomato (ripe)	"	5·7γ
	40	Plantain (raw), *Musa sapientum*, H Kachakela, B Kanchakala, Tm Valakkaui T Arntikaya, M Valakkey, C Balachanui, O Konchakodoli	Malabar	5·6γ
	41	Plantain (ripe), H Pakkakela, B Kala, Tm Valapalam T Aratipandu, M Valapalam C Valapal, O Pachola kodoli	"	9·1γ
	42	Drum stick, *Muringaceæ Sp*, H and B Sajna, Tm Muringa kaui, T Mungi kaya, M Murngakka, C Nuggekai O Sojona	"	17·5γ
	43	Drum stick plant leaves, H Sajna sag, B Sajna sak, Tm and M Murngikeerye, T Murngi Kora, C Nuggekeera, O Sojona sag	Coimbatore District	51·0γ
	44	Lady finger, *Hibiscus esculentum*, H Ramtorai, B Dharaush, Tm Vendakaui, T Vendakaya, C Vendakai, M Vendakai O Bhendi	"	6·8γ
	45	Amaranth, *Amarantus gangetus*, H Bathu B Lalsak Tm Thandukeerye, T Thotakora, M Thandukeerai, C Thadukeerni O Khodn or Kosolo sag	"	56·3γ

No.	Description	Source	Value
46	Amaranth perennial, *Amarantus cadatus*, H Palack, B Ananta mool, Tm Sirikeery, T Kokoru, M Sirukkere, C Chnuk kererai, O Neguna	Coonoor	27 1γ
47	Prickly amaranth, *Amarantus spinosus*, H Karund, B Kanta sak, Tm, M and C Mullukeerye, T Mullukoru, O Konta marisi	,,	11 3γ
48	French bean, *Phaseolus vulgaris* H Mutter, B Mottor or Sim, Tm Beans, T Seemachkudikava, M Vilathi avaraka, C Vilathi chettu, O Bilathi simo	,,	13 6γ
49	Soya bean, *Glycine hispida*	Market sample	9 0γ
50	Cluster bean, *Cyamopsis psoraliodes*, H Mutter, B Mottor, Tm Kothavarangau, T Goruchkudikaya, M Kothavarathka, C Govardhankai, O Gobardhanjhota	Combatore District	11 3γ
51	Broad bean, *Vicia faba*, H Sim, B Sim, Tm Bean, T Pedda chikudikava, M Veedipyaru, C Soppavaray, O Tamuko jhota	,,	9 0γ
52	Sesbania beans *Sesbania grandisflora* H Agasthphali, B Agasthim, Tm Avithkau, T Avithkora, M Avithkka, C Aiskai, O Ogosthpholo	,,	4 7γ
53	Sesbania leaves	,,	22 5γ
54	Cucumber, *Cucumis sativus*, H Khira, B Sawsa, Tm Velirikaui, T Dosakaya, M Velrikka, C Sonthikai, O Kakudi	Malabar	5 0γ
55	Pumpkin or sweet gourd, *Cucurbita maxina* H Halwa kodu, B Mitaha kodu, Tm Pusankai, T Gumudikaya, M Mattanga, C Kumbulakai, O Bothikokharu	Combatore District	9 8γ
56	Water gourd, *Benincesa cerifera*, H Petaha, B Chalkumda, Tm Tanni swarakaui, T Puchakaya, M Mathan swaraka, C Heirekai O Panikokharu	,,	6 8γ
57	Gourd, *Lagenaria vulgaris* H Lokee or Kodu, B Lau, Tm Swarakkaui, T Anapakaya, M Sworakka, C Sworakkai, O Lau	,,	4 5γ

TABLE I—contd

Group	Serial number	Sample	Source	Iodine content per kg
VI. Garden produce and green vegetables—concld	58	Snake gourd, *Tricosanthes anguina*, H Thar, B Sachinda, Tm Pudalungkaui, T Potolokaya, M Podalanga, C Padalakai, O Sapua	Coimbatore District	4 5γ
	59	Ridge gourd, *Luffa acutangula*, H Thori, B Jhinga, Tm Peerungkaui, T Beerikaya, M Peerikinga, C Peerekai, O Janhi	,,	11 3γ
	60	Bitter gourd, *Memordica hroca*, H and B Karala, Tm Pavakaui, T Kakarkaye, M Kappakya, C Haugalkai, O Kolora	,,	6 8γ
	61	Cauliflower, *Brassica rapa*, H Phulgobi, B Phulkopi, Tm, M and C Cauliflower, T Kobbipoovu, O Phulokobi	Nilgiris	9 0γ
	62	Summer cabbage, *Brassica oleracea*, H Gobi, B Bandakopi, Tm, M and C Muttre goves, T Kobbi, O Patharkobi		
		(*a*) Innermost leaves	,,	9 5γ
		(*b*) Outermost leaves	,,	77 7γ
	63	Winter cabbage (*a*) Innermost leaves	,,	9 0γ
		(*b*) Outermost leaves	,,	14 0γ
	64	Lettuce, *Lactuca scariola*	,,	58 0γ
	65	Ipomea, *Ipomea reptans*, B Kalmisak, Tm Parupukeerye, T Papukora, M and C Parupa keerai, O Kolomi sag	,,	27 0γ
	66	Spinach, *Spinacia oleracea* B Palang sak, Tm Pulchikeerye, T Puntikora, O Palango sag	Coimbatore District	40 6γ
VII Condiments and spices	67	Onions, *Allium cepa*, H Piaj, B Piani, Tm Vengayam, T Uliigadda, M Chuvannauli, C Nirulli, O Piajo	Malabar	0 8γ

No.	Name	Source	Value
68	Garlic, *Allum sativum* H Lasan, B Roshoon, Tm Vellaipoondu, T Tella ulli, M Vellulli, C Bellulli, O Roshno	"	27 0γ
69	Ginger, *Zingiber officinale*, H Adrack, B Ada, Tm Inji, T Allum, M Inchi, C Hassunti, O Oda	"	124 3γ
70	Turmeric *Curcuma longa* H Holdi, B Holood, Tm and M , Mayal, T Pasupu, C Arashna, O Holdi	"	22 5γ
71	Asafœtida, *Ferula asafœtida*, H and B Hing, Tm Perungayam, T Hingu, M Kayam, O Hingu	Market sample	nil
72	Black pepper, *Piper nigram*, H Kalamirch, B Golemarich, Tm Milagu, T Miralu, M Kurumulagu, C Ollemunasu, O Golemoricho	Malabar	90 2γ
73	Chillies (ripe and dry), *Capsicum annum* H Mirch, B Lonka, Tm Milakaui, T Merupukaya, M Molaku, C Menasunkavi, O Lonka morieho	"	22 5γ
74	Chillies (raw)	'	4 5γ
75	Cardamom, *Eletheria cardamomum* H Ilachi, B Ilach, Tm Elakkaui, T Yelakkavulu, M Elum, C Yalaki, O Olaicho	Market sample	22 5γ
76	Cumin, *Cuminum cyminum*, H Jeera, B and O Ira, Tm Seeragam, T Jilkrara, M Jirakam, C Jirge	Malabar	135 5γ
77	Coriander, *Coriandrum sativum*, H, B and O Dhania, Tm Kothumalli, T Kothumri, M Kothamphari, C Kothum bari	"	67 6γ
78	Fenugreek, *Trigonella fœnugacum* H Methi, B Methi, Tm Ventluam, T Menthulu, M Uluva, C Menthia, O Methi	"	nil
79	Cloves, *Engenia caryophyllata*, H Lavang, G Lovongo, Tm Lavangam, T Lavangalu, M and C Lavang O Lovongo	"	507 2γ
80	Common salt, H Ni nak, B Lovon, Tm, T, M and C Uppu, O Nuno	"	180 0γ

TABLE I—*concld*

Group	Serial number	Sample		Source	Iodine content per kg
VIII. Dairy, fishery, poultry and farm products	81	Colostrum		Coonoor	30 4γ (*per litre*)
	82	Cow's milk		,,	39 8γ (*per litre*)
	83	Goat's milk		,,	92 4γ (*per litre*)
	84	Buffalo's milk		,,	34 0γ (*per litre*)
	85	Butter		,,	30 1γ
	86	Cod liver oil		,,	5,505 0γ (*per litre*)
	97	Sea prons (fresh)		Calicut	1,121 0γ
	88	Sardines (fresh)		,,	479 0γ
	89	Duck egg	(a) white	Coonoor	58 6γ
			(b) yellow	,,	164 0γ
	90	Fowl egg	(a) white	Nutritional Research, Coonoor	67 6γ
			(b) yellow	,,	158 0γ

91	Meat (sheep)	Market sample	18 0γ
92	Liver (sheep)	"	32 7γ
IX Miscellaneous food products			
93	Vitamin A concentrate	Nutritional Research, Coonoor	9 0γ (per litre)
94	Vitamin B complex (yeast powder)	"	39 8γ
95	Jagery, H and B Goor, Tm Vellum, C Bella, T Bellamu, O Gudo	Coimbatore District	22 6γ
96	Cane sugar, *Saccharum officinarum* H and B Cheeni, Tm Sakkarye, T Panchdar, O Nobatho	Market sample	nil
97	Coffee powder (roasted), *Coffea arabica*	Nilgiris	nil
98	Betel leaves, *Piper betel*, H B and O Pan, Tm Vethlye, T Thamalapakulu, C Vihdele	Coimbatore District	33 8γ
99	Tobacco (dry), *Nicotiana tabacum*, H and B Tamak Tm Pugaile, T Pogaku, M Pukayla, C Hogesoppu, O Dhnau pothoro	"	169 0γ
100	Tap water	Coonoor	0 45γ (per litre)

The following variations in different samples of the same food-stuff were noted rice from 18 1γ to 31 6γ per kg , wheat from 22 8γ to 27 0γ , gram from 22 5γ to 33 8γ , mango from 9γ to 16 4γ , plantain from 5 6γ to 9γ , amaranth from 11 3γ to 56 3γ , beans from 4 7γ to 11 3γ , and gourds from 4 5γ to 11 3γ A remarkable result is that the iodine-content of the outer leaves of cabbage is different in winter from that in summer , being much greater at the latter season (77 7γ as compared with 14γ) Further, the inner leaves of cabbage contain much less iodine than the outer leaves during the summer but not during the winter months an observation which may have a bearing on the seasonal variation noted in the goitrogenic potency of cabbage (McCarrison, 1931)

It is further to be noted that many condiments in use in India are rich in iodine, notably ginger, cumin, coriander, black pepper and cloves Eggs are relatively rich in iodine, the yolk containing more than twice as much as the white It is to be noted also that goat's milk contains more than twice as much iodine as cow's or buffalo's milk Yeast is relatively rich in iodine, while a proprietary preparation of vitamin A was found to contain traces of it One interesting finding is that tobacco contains relatively large amounts of iodine

Comments on the relation of the iodine-content of certain Indian diets to the occurrence of goitre

From the above estimations it is possible to compute the approximate iodine-contents of certain national diets of India that of the Sikhs, Mahrattas, Pathans, Gurkhas, Canerese, Bengalis and Madrasis (McCarrison, 1927) These computations are as follows :—

TABLE II

Giving the approximate iodine-content of certain national diets of India

Diet number	Diet	Calorific value (calories)	Iodine content per head per day
1	Sikh diet	2 670	30γ
2	Mahratta diet	2,560	36γ to 66γ or 51γ
3	Pathan diet	2,860	23γ
4	Gurkha diet	2,870	35γ to 70γ or 52 5γ
5	Canarese diet	2,600	55γ
6	Bengali diet	2,680	40γ
7	Madrasi diet	2,600	28γ

It will be noted that in diet Nos 1, 3, 6 and 7 the iodine-content does not reach the level (45γ) stated by Orr and Leitch (1929) to be necessary for safety , yet goitre is very rare in Madras and in parts of Bengal (McCarrison, 1915)

Further data relating to the iodine-intake of Madrasi children, living in this neighbourhood (Coonoor) and who are free from goitre, are as follows :—

TABLE III

Giving the approximate iodine-intake of Madras children in the neighbourhood of Coonoor

Age	Diet number	Description of diet	Calorific value (calories)	Daily iodine intake in γ
1½ years	8	Rice 120 g, milk 350 c.c., vegetables and fruits 50 g, onion, salt and condiments 2 g, a little water	1,060	37
,,	9	Rice 130 g, milk 300 c.c., biscuits and sweets 50 g, dhal 10 g, vegetables 20 g, salt and condiments 2 g, a little water	930	18
6 ,,	10	Vegetarian diet Rice 350 g, vegetables 200 g, roots 20 g, dhal 25 g, oils 10 g, fruits 50 g, condiments and spices 5 g, and water	1,680	18
,,	11	*Non vegetarian diet* Rice 350 g, vegetables 150 g, roots 20 g, dhal in the form of sweets 20 g, oils 10 g, sugar 10 g, fish, meat or eggs 15 g, condiments and spices 8 g, and water	1,600	17 to 27 or 22
12 to 14 years	12	*Vegetarian diet* Rice 650 g, roots 50 g, vegetables 250 g, dhal 50 g, oils 20 g, buttermilk 100 c.c., fruits sparingly, condiments 10 g, and water	2,870	29
,,	13	*Non vegetarian diet* Rice 650 g, roots 50 g, vegetables 150 g, dhal 20 g, oils 20 g, buttermilk and fruits sparingly, fish, meat or eggs 50 g to 100 g, condiments and spices 20 g, and water	2,800	26 to 96 or 61
15 to 18 years	14	*Vegetarian diet* Rice 780 g, roots 100 g, vegetables 200 g, dhal 50 g, oils 40 g, buttermilk 100 c.c. to 200 c.c., sugar 25 g, fruits sparingly, onions, spices and salt 25 g, and water	3,310	33
,,	15	*Non vegetarian diet* Rice 780 g, roots 50 g, vegetables 150 g, dhal 25 g, oils 40 g, sugar 25 g, fruits sparingly, onions, condiments and spices 50 g, fish, meat or eggs 50 g to 100 g, and water	3,740	33 to 103 or 68

These observations show that the iodine-intake of infants, living in the neighbourhood of Coonoor, varies from 18γ to 37γ per day and that of school children up to the age of six years from 18γ to 27γ They further show that boys and girls between the ages of 12 and 18 years, living on vegetarian diets, consume only 29γ to 33γ of iodine daily, while the intake in non-vegetarians varies from 26γ to 103γ

If the minimum requirement of iodine is, indeed, that laid down by Orr and Leitch, namely, 45γ for adults and 150γ for children of school age, then it is apparent from the above data that the intake of iodine by many children and adults in India falls far short of these requirements In the Presidency of Madras, for example, the iodine-intake of the average adult—as in my own case—is only 28γ Further, the incidence of goitre in the vegetarian boys of Coonoor, whose age is round about fourteen, is nil, although their consumption of iodine is, on an average only 31γ per day From these observations it is apparent that the intake of iodine in amounts considerably less than those reputed to be necessary for the prevention of goitre is not associated with the occurrence of the disease in people of this locality (Coonoor)

This investigation was undertaken at the suggestion of Sir Robert McCarrison and under his direction I desire to express my indebtedness to him for his continued help and advice throughout its course

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SOME COMMON INDIGENOUS REMEDIES

BY

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In this paper we have given a brief description of the studies carried on by us during the past 2 or 3 years on the chemical composition, pharmacological action and therapeutic properties of some of the common remedies used in Indian indigenous medicine   Although these plants are largely used in many parts of India, our studies show that they do not contain sufficient quantities of physiologically active substances to produce marked therapeutic effects   In some cases bitters and traces of alkaloids have been found   In this paper we have given a brief description embodying the result of our investigations

PICRORHIZA KURROOA, BENTH

(With A T DUTT, b sc)

This plant belongs to the Natural Order *Scrophularineæ* and grows throughout the Alpine Himalaya from Kashmir to Sikkim   It is a low herb with a perennial woody, bitter stock   The root stock is nearly as thick as the little finger, 6 to 10 inches long   The plant is known in Sanskrit as *Katuka*, in Bengali and Hindi as *Katki*, in Tamil as *Katuka-vogani*, in the Punjab as *Karru*, in Guzerat as *Kadu* and in Bombay as *Balakadu*   The root is described as bitter, stomachic and in large doses cathartic   It is used in fever, dyspepsia and in nervous pain of the stomach and bowels   It is also used as an antiperiodic and a stomachic tonic   Moodeen Sheriff recommends a strong decoction of the drug 3 or 4 times a day continued for 3 or 4 days in cases of dropsy   It produced copious watery evacuations and

decreased œdema of tissues   It has also been recommended in the treatment of malarial fever   The drug was admitted into the Indian and Colonial Addendum of the British Pharmacopœia

Tho roots were examined chemically by Dymock, Warden and Hooper (1893) and they isolated a wax, a bitter principle, which they designated as *Picrorhizin*, cathartic acid and other organic acids   Later, Ghoshal (1912) examined the drug and studied its pharmacological action in detail He stated that the action of tho drug was due to the bitter principle, that it had no poisonous action and that it increased the gastric secretion   It also diminished the force of the heart beat and hence might bo used in febrile cases, tho beneficial effect being due to the reduction of blood pressure   It had a mild laxative action due to the presence of cathartic acid

As it is commonly used in indigenous medicine, we re investigated its chemical composition and isolated the bitter principle in a pure state

Chemical examination —The powdered root was extracted successively with petroleum ether, sulphuric ether, absolute alcohol and water, the solvents were removed and the residue dried and weighed   Petroleum ether extracted 1 49 per cent, sulphuric ether 3 45 per cent, absolute alcohol 32 42 per cent and water 8 46 per cent of tho weight of dry powder   The petroleum ether extract contained a fatty oil, a waxy substance, phytosterol and faint traces of a basic substance   The sulphuric ether extract contained organic acids, small amounts of a bitter substance and 1 79 per cent of a resinous substance   The alcoholic extract contained besides tho main bulk of the bitter substance, organic acids and resinous substances   The aqueous extract contained sugar and inorganic substances containing a fair amount of calcium salts   No alkaloid could be detected in the other extracts

Isolation and purification of the bitter substance (a glucoside) —Tho powdered root was extracted continuously with rectified spirit in a percolator at room temperature until exhausted   The alcoholic extract was mixed with some $CaCO_3$ and tho alcohol recovered under reduced pressure   The residue was then dissolved in water, filtered and the filtrate was first precipitated with neutral lead acetate   The precipitate was filtered off and the filtrate was precipitated with basic lead acetate   The latter precipitate was filtered, washed thoroughly and dried   The dry precipitate was finely powdered suspended in absolute alcohol and decomposed by H_2S The filtrate from PbS was freed from H_2S by a current of CO It was filtered and the alcohol was removed from the filtrate by concentration *in vacuo*   The residue was thoroughly dried in a vacuum desiccator   It was then dissolved in absolute alcohol and precipitated with dry ether   The process was repeated 3 or 4 times until the bitter glucoside was obtained as a cream coloured amorphous powder   We could not obtain it in a crystalline state   The yield was nearly 26 6 per cent   The bitter glucoside was freely soluble in alcohol, acetone, acetic ether and hot water   It was fairly soluble in cold water but insoluble in ether, chloroform benzene and petroleum ether   It had no definite melting point and was very hygroscopic   It showed a specific rotation, $[a]_D^{20} = -100°$ in aqueous solution   Concentrated H_2SO_4 produced a deep brown coloration which changed to blood red and concentrated HNO_3 produced a deep yellow colour   On hydrolysis it produced about 31 per cent of an insoluble black substance and the sugar produced on hydrolysis was identified as glucose by the melting point of the osazone found   The determination of tho molecular weight by the elevation of tho boiling point of alcohol did not lead to any definite conclusion   The elementary analysis gave the following figures —

| Carbon | 53 37, | 53 20, | 53 13, | 53 23 |
| Hydrogen | 6 69, | 6 73, | 6 92, | 6 82 |

It did not contain any nitrogen

The glucoside was acetylated with acetic anhydride   The acetyl derivative showed the melting point 81 8°C   Its weight by tho freezing point depression in benzene gave the average value of 695

Pharmacological action —The pharmacological action of the pure glucoside was investigated but it showed no marked physiological activity   The decrease in the force of the heart-beat observed by Ghoshal (*loc cit*) could not be obtained unless very large doses of the glucoside were administered   Picrorhiza root contains considerable quantities of this bitter glucoside and its therapeutic effect like that of gentian depends much on this bitter principle   It can form a good substitute for gentian and other bitters in use in the pharmacopœias of western countries

ERYTHRINA INDICA

(WITH B N SEN)

The plant is known as the Indian coral tree, *Parijata* (Sanskrit), *Pangra* (Hindi), *Palita-mandar* (Bengali), *Kaliyana murukku* (Tamil), *Pangara* (Mar), *Pararoo* (Gur) and belongs to the Natural Order *Leguminosæ*   The tree is 9 to 18 feet high and grows all over India and bears red flowers in the month of February and March The bark has small fissured corky lenticles arranged in perpendicular rows   On rubbing off the superficial layer a green surface is exposed   The outer portion of the bark is granular and brittle, the inner consists of numerous interlaced layers of outer liber cells so arranged as to form a network

The plant has long been known to Hindu physicians for its medicinal properties   The leaves, juice and the bark are used in medicine   The leaves are said to be laxative, diuretic, aphrodisiac, galactagogue and emmenagogue   With coco-nut milk they are used as a galactagogue   They are used to kill worms in sores and are applied externally to disperse venereal buboes and to relieve pain in the joints   It is an anthelmintic and is useful as a collyrium in conjunctivitis   The bark is astringent and is given in dysentery and fever, it is also said to have febrifuge and antibilious properties   Core and Lejaune (*Resume de la Mat Med Coloniale*) stated that the bark is expectorant and febrifuge, the leaves laxative and diuretic   The first physiological experiment was made by Bochefontaine and Rey (1881) who arrived at the conclusion that the drug acts upon the central nervous system so as to diminish its functions

According to Dymock, Warden and Hooper (1890) the bark contains two res ns, the one soluble and the other insoluble in dilute alkalis and a bitter alkaloid   An alkaloid giving a crystalline hydro chloride was isolated by Allamirano (1889) in the same tree growing in Mexico and he recommended it as an antidote against strychnine   According to this worker with previous administration in six decigram doses of erythine poisonous doses of strychnine could be taken without danger

As the bark is largely used in indigenous medicine we re examined it to isolate any active principle to which the above medicinal value might be attributed

*Chemical composition —*An assay of the total alkaloids in the bark showed the presence of 0 05 per cent of alkaloids   Five hundred g of the bark were successively extracted with petroleum ether (B P 35°C to 70°C), ether and absolute alcohol and after distilling off the solvents the extracts were dried in a desiccator, weighed and separately examined   Petroleum ether extracted 0 60 per cent , ether extracted 2 03 per cent and absolute alcohol extracted 3 14 per cent

The petroleum ether extract was re dissolved in 300 c c of petroleum ether (35°C to 70°C) and shaken with water, acidulated water 5 per cent sodium carbonate solution and 5 per cent caustic soda solution   The aqueous extract did not show the presence of tannins (ferric chloride test), glucosides or carbohydrates (with α–naphthol and sulphuric acid)   The acid aqueous extract did not give any test for alkaloids   The Na_2CO_3 and NaOH extracts were acidified and shaken with petroleum ether from which some fatty acids semi solid at the ordinary temperature, were obtained   After it was subjected to the above process the petroleum ether solution of the petroleum ether extract gave some fatty oils

The ether extract was treated in the way described above and showed the presence of chlorophyll and traces of alkaloids

As the absolute alcoholic and 70 per cent alcoholic extracts showed the presence of similar sub stances both were worked together   It was found that from the absolute alcoholic solutions, substances separated on cooling which proved to be KCl and K_2CO_3 The joint extracts were then repeatedly shaken with one per cent hydrochloric acid till exhausted of alkaloids and on adding more acid resins separated which were filtered off   The solution was neutralized, filtered, excess of ammonia added and repeatedly shaken with chloroform   A quantity of impure alkaloid was thus obtained as a dark coloured varnish like mass   The aqueous portion, still showing the presence of bases, was shaken with ether, benzene and amyl alcohol but still it gave tests with alkaloidal reagents   Water soluble bases were thus present

For the isolation of the water soluble bases, 11 5 kilos of the bark were extracted with 92 per cent alcohol and after the major portion of the solvent had been distilled off the remainder was distilled under vacuum at a temperature below 70°C   A dark brown mass was obtained from which the alkaloid was extracted described as under the absolute alcoholic extract   Next the aqueous portion was neutralized with soda solution, concentrated to 500 c c under vacuum and the bases precipitated with phosphotungstic acid   The bases were liberated by keeping the phosphotungstates in fine suspension in water and adding excess of baryta solution   The precipitate of barium phosphotungstate was filtered off at the pump and excess of Ba removed by passing carbon dioxide into the filtrate   The dark red filtrate was treated with basic lead acetate to give a precipitate which was rejected and the filtrate was deleaded with H_2S and the H_2S removed by passing CO_2 The filtrate was concentrated to dryness in vacuum thus removing most of the acetic acid and was shaken with 200 c c of absolute alcohol   The absolute alcoholic solution was precipitated with alcoholic mercuric chloride, choline and betaine was precipitated and the filtrate contained some unidentified bases

The mercury compound was decomposed with H_2S, the sulphide precipitate removed and the filtrate containing choline and betaine was concentrated to dryness and shaken with cold absolute alcohol   Betaine hydrochloride being insoluble in absolute alcohol was thus separated and recrystallized in colourless leaflets melting with decomposition at 227°C to 228°C   A solution of the hydrochloride when added to a mixture of potassium ferricyanide and ferric chloride solution turned it greenish blue (test for betaine)   When a solution of platinic chloride containing sodium iodide was added to a solution of the hydrochloride dark coloured rectangular crystals amongst which a few were truncated were observed under the microscope (test for betaine)

From the filtrate choline chloride, very hygroscopic and readily soluble in alcohol, crystallized in flat colourless needles   The platinic chloride crystallized in orange yellow regular system (octahedra)   Choline chloride gave with iodine and mercuric iodide a crystalline precipitate and formed a gold chloride in tufts of yellow needles and was thus identified

5 71 g or 0 05 per cent of crude total alkaloid was obtained from 11 5 kilos of the bark   It was extracted with 2 per cent tartaric acid, the acid solution made alkaline with ammonia avoiding excess and shaken with chloroform   It was found that the alkaloid dissolved in excess of ammonia or NaOH and was then extracted with difficulty with chloroform   On distilling off the chloroform 3 25 g of pale yellow coloured varnish like alkaloid was obtained   An amorphous alkaloid melting at 97°C with decomposition has, however, been isolated from a precipitated citrate but it was almost tasteless and was not physiologically active

Briefly the following substances were found in *Erythrina indica*

1   Resins (major portion of the alcoholic extract)

2   Fixed oils and fatty acids 0 60 per cent

3   Alkaloids to the extent of 0 05 per cent from which an alkaloid decomposing at 97°C was obtained

4   Water-soluble bases in appreciable amount amongst which betaine and choline were isolated and identified

5   Salts, chiefly KCl and K_2CO_3

Pharmacological action —The alkaloid and the different fractions obtained during the course of analysis were tested pharmacologically but no remarkable activity was observed   In the absence of any signs of physiological activity clinical trials were not carried out

SANSIVIERA ZEYLANICA

(With A N RATNAGIRISWARAN)

This plant belongs to the Natural Order *Liliaceæ* and grows on the Coromandel coast and in Ceylon   It is known in Hindi as *Murahri*, in Sanskrit and Bengali as *Murbá*, in Tamil as *Márul-kálang*, in Guzerati as *Murvel* and in Mal as *Katu-kapel*   It is described as a purgative, tonic and cardiac stimulant and is used as a remedy in heart disease, itch, leprosy, rheumatism, etc   According to Ainslie the root is

prescribed in the form of an electuary in consumption and allied pulmonary affections   The plant yields a good fibre and on this account is cultivated in some parts of the Madras Presidency   The juice of the tender shoots of the plant is administered to children to clear their throat of viscid phlegm

Dymock, Warden and Hooper (1893) examined the roots and isolated a wax, salicylic acid, a neutral bitter resin and a crystallizable alkaloid, which they named *Sansevierine*   To confirm their results and to isolate, if possible, the crystalline alkaloid in sufficient quantities for investigation of its pharmacological action assays were made both by B P and U S P methods   Two such assays showed the presence of only 0 018 per cent of total alkaloids in the plant

A larger quantity of the plant was next extracted with acidified alcohol and the alkaloid was isolated and purified in the usual way, but the quantity of the alkaloid obtained was very small   It would appear from the results obtained that the drug could not possess any marked degree of therapeutic activity

PONGAMIA GLABRA

(With N R CHATTERJEE)

This is a handsome flowering tree with foliage like the beech, with drooping branches of shining green leaves, laden with racemes of rose-coloured flowers   It grows widely in India and is more abundant near the coast   It is known in Sanskrit as *Karanja*, in Hindi as *Karni*, in Bengali as *Dahar Karanja*, in Tamil as *Pungammaram*, in Mahratti as *Kidamar* and in the Punjab as *Sookchein*   The seeds, the leaves and the oil are used in Hindu medicine as a remedy for skin diseases and rheumatism and to destroy worms in sores   The juice of the root is used for cleaning foul ulcers   The oil is used for application to rheumatic joints and as a remedy for scabies, herpes and other cutaneous diseases   It forms ingredients in several complicated prescriptions for epilepsy

We received a sample of the seeds from Hyderabad with the request to analyse them as they had a great reputation as a remedy for whooping cough

The pods are generally 1½ to 2 inches long containing one perfect seed   The seed is compressed, of the shape and size of a bread bean , testa thin, smooth, light red , cotyledons very oily

According to Lepine (1860) the seeds yield 27 per cent of an yellow oil, having a sp gr of 0 945 and solidifying at 8°C   The oil expressed from fresh seeds and examined by Hooper (Dymock *et al*, 1890) was thick of a light orange brown colour, and bitter taste   The sp gr at 18°C was 0 9358   The bitter taste of the oil appears to be due to the presence of a resin and not an alkaloid

The bark, examined by Hooper (Dymock *et al*, 1890), is said to contain a bitter alkaloid, soluble in ether, alcohol and water   also an acid resin of a greenish brown colour soluble in ether   The alcoholic extract is composed of a substance analogous to quinovin together with sugar   The watery extract contains much mucilage, which is gelatinized by ferro chloride   No indication of the presence of tannin could be obtained from any part of the bark

In his textbook Lewkowitsch (1922) describes the physical and chemical characteristics of the pongam oil as well as of those of the insoluble fatty acids obtained from it   In another report Desai, Sudborough and Watson (1923) have described the isolation of myristic, palmitic, stearic, arachidic, lignoceric and dihydroxy stearic acids from the oil

We extracted about 1 kilo of the powdered seed kernels in an extraction apparatus successively with petroleum ether, ether and alcohol   The solvents were removed in each case and the residue examined for the constituents   The petroleum ether extract yielded a pale brown fatty oil over 30 per cent of the weight of the kernels   As they have been examined thoroughly they were not tested further

The ethereal extract was re dissolved in ether, when a portion did not dissolve readily   It was found to be a resin acid   The portion in ethereal solution did not yield anything of importance or interest

The alcoholic solution, on keeping deposited a few crystals   These crystals were separated from the mother liquor, washed with alcohol and tested   It was identified to be pure cane sugar   The alcohol was then removed and the residue treated with water   The aqueous solution gave strong

reaction for carbohydrates but none for alkaloids   An ethereal and ethyl acetate extract of the aqueous solution gave nothing definite

A quantity of the powdered drug was moistened with water and distilled in a current of steam A very small quantity of an essential oil was obtained   This is the only active principle obtained which might produce relaxation of the spasm of bronchial muscles occurring in whooping cough

HYGROPHILLA SPINOSA

(WITH A N RATNAGIRISWARAN)

This is an annual marshy herb with an ascending rhizome, belonging to the Natural Order *Acanthaceæ*   It is met with throughout India from the Himalayas to Ceylon in all inundated areas and in the riverways   It is known as *Ikshura Kohilaksha* or *Kashura* in Sanskrit, *Kuhahara* in Bengali, *Tal-makhana* in Hindi, *Numalli* in Tamil and *Ekharo* in Guzerati

In Hindu medicine the plant is a favourite remedy against urinary disorders The leaves are described as cooling and useful in jaundice and ascites   The root is considered as a bitter tonic and is used in rheumatism and urinary affections The seeds are given for gonorrhœa   They contain a large quantity of tenacious mucilage   The root is considered to be cooling, bitter and tonic, it has diuretic properties and is frequently employed in dropsical cases and against rheumatism Many European physicians who have tried the root bear testimony to the diuretic properties of the root   It will be significant to note in this connection that the ashes of the plant are also used for the same diseases for which the fresh root and seeds are prescribed, as for instance in hepatic obstruction with dropsy and urinary affections   It, therefore, seems quite likely that the medicinal properties of the plant are dependent partly, if not wholly, on its inorganic constituents

Preliminary examination for alkaloid gave tests of doubtful value and, therefore, a large quantity (about 4 kilos) of the dry plant cut into small pieces, was extracted by continuous percolation with alcohol and the residue left after evaporation of the solvent submitted to the usual examination

A basic amorphous residue weighing only 0 12 g and giving marked reactions with the alkaloidal reagents was obtained but owing to the very low yield of this constituent no attempt was made to study it further

The aqueous alkaline solution after removal of the alkaloid was precipitated successively with lead acetate and basic lead acetate, and the precipitates and the final filtrate examined for glucosidic compounds but with negative results   The residue from the final aqueous filtrate, freed from tannin, colouring and other matters precipitable by lead sub acetate, consisted mainly of potassium salts and some sugars

The diuretic and the soothing properties of the drug are due to potassium salts and to the large quantities of mucilage it contains

BRYOPHYLLUM CALYCINUM, SALISB

(WITH A T DUTT)

This plant belongs to the Natural Order *Crasulaceæ* and is found throughout India   It is very common in lower Bengal   It is known in Bengali as *Kop pata*, in Bombay as *Ghaimari* and in Tamil as *Malaikalli*   It is a perennial succulent herb with succulent ovate or oblong leaves, 3 to 6 inches in length   The margin of the leaves and the petioles are coloured purple   The leaves roasted are used as an application to wounds, bruises, boils and bites of venomous insects   The juice of

the leaves is used in dysentery   It is also used in the form of poultice and powder for sloughing ulcers

Dymock, Warden and Hooper (1890) report the presence of fat, an organic acid, calcium oxalate, acid potassium tartrate etc in the leaves

The air dried leaves and stems were powdered and extracted successively with petroleum ether, ether chloroform and absolute alcohol, the solvents were removed and the residues weighed   Petroleum ether extracted 2 22 per cent of the dried plant and contained some fatty oil, ether extracted 0 91 per cent and contained some organic acids and a resin, chloroform extracted 0 43 per cent and contained a caoutchouc like substance and absolute alcohol extracted 1 83 per cent of the dry plant and contained phlobaphenes and a very small quantity of an alkaloid   An assay for the estimation of alkaloids was done in duplicate and showed the presence of only 0 008 per cent of the alkaloids As the quantity of the alkaloid was very small and as there was no indication of the presence of any other pharmacologically active constituent, the work was not pursued further

RHEUM EMODI

(With N N GHOSH)

The plant belongs to the Natural Order *Polygonaceœ*   It is found in the western Himalayas and Kumaon   It is known in Hindi and Bengali as *Revand-chini*, in Tamil as *Variyathu*, in Nepal as *Padamchal*, in Guzerat as *Gamniravan chini* and in Mahratti as *Mulkacha reval chini*   Though not mentioned in the Hindu system of medicine, *Rheum emodi* (Rhubarb) is commonly used in India as a stomachic tonic and mild aperient   It occurs in twisted or cylindrical pieces about 4 inches long and $1\frac{1}{2}$ inches in diameter   It is of a dark brown colour and a bitter astringent taste Several species of rhubarb grow at higher altitudes in the Himalayas, e g , (i) *Rheum emodi*, (ii) *R moorcroftianum* and (iii) *R webbianum*   Considerable quantities are annually conveyed from the Kangra district of the Punjab for use in indigenous medicine

The sample under examination was that of *Rheum emodi* and was kindly supplied by the Conservator of Forests Utilization Circle, Srinagar   It gave the following results on analysis   Total alcoholic extract, 32 per cent , emodin, about 0 15 per cent , total cathartic acid, 3 35 per cent , fat and chrysophanic acid, 1 75 per cent   ash, 7 30 per cent   Foreign rhubarbs imported into India generally contain about 3 5 per cent of cathartic acid which is supposed to be the purgative principle in rhubarb The sample under examination thus compared very favourably with the foreign samples

SOLANUM INDICUM

(With A T DUTT)

This plant belongs to the Natural Order *Solanaceœ* and is very common throughout tropical India   It is an erect shrub, very prickly, with yellow berries about half an inch in diameter   It is known in Sanskrit as *Vrihati*, in Hindi as *Barhanta*, in Bengali as *Byakura*, in Tamil as *Pappramulli*, in the Punjab as *Kandyari* and in Bombay as *Dorli*

The roots, leaves and berries are used in medicine   The roots constitute an ingredient of *Dashamula*   It is prescribed by Hindu physicians in the treatment of dysurea and is regarded as an expectorant and useful in cough and catarrhal affections   It is also used in difficult labour, fevers, etc

The plant was examined chemically by Dymock, Warden and Hooper (1891) who found the presence of an oil and traces of an alkaloid answering to solanine   In the seeds they found an alkaloid probably different from solanine and also the presence of a glucosidic principle   Many chemical investigations have been carried out later on with various plants belonging to the genus *Solanum* and

tho alkaloids found in them have been thoroughly investigated. We made a preliminary chemical examination of the Indian plant available locally in order to determine if thero was sufficient amount of the total alkaloids for a detailed examination of the individual constituents

Tho fresh green berries were dried and were found to contain about 83 4 per cent of water. The dried berries were powdered and extracted successively with different solvents, the solvents were removed in each case and the residue weighed. Petroleum ether extracted 6 33 per cent, sulphuric ether 1 82 per cent, chloroform 2 23 per cent and absolute alcohol 9 47 per cent of the dried berries. The petroleum ether extract contained some fatty oil and sulphuric ether extract contained a small quantity of glucosidal substance and some organic acids. The chloroform extract showed traces of an alkaloid and glucosidal substance. The absolute alcoholic extract contained the glucoside and alkaloids. Assays of the entire dry plant (including leaves, stalk and berries) showed the presence of only 0 02 per cent of alkaloid. An assay in duplicate for the alkaloids in the dried berries showed that they contained only 0 1 per cent of total alkaloids

Pharmacological action —The action of solanine has already been investigated. It resembles certain of the non-alkaloidal glucosides, such as the saponins, but it is much less powerful

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BIOLOGICAL ASSAY OF DIGITALIS PREPARATIONS IN THE TROPICS

Part IV

BY

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IN a previous paper (Chopra and Chowhan, 1933) we have remarked that the standards and methods of biological assay adopted in temperate climates are not always suitable for tropical climates such as that of India   The frog method has not been found practicable in Calcutta, though Bhatia and Lal (1934) found it satisfactory in Lucknow   After trials on a number of laboratory animals we have come to the conclusion that Chopra's modification of Hatcher and Brody's cat method, details of which have been previously published, has given generally satisfactory results   Although observations made in America on digitalis tinctures show that they do not appreciably change within a period of two years, we have shown (Chopra, Bose and De, 1925) that climatic conditions and condition of storage in India undoubtedly produce a rapid change in tinctures which become therapeutically inactive and sometimes toxic from the point of view of biological assay   Bose (1925) in the course of his clinical studies on digitalis observed that the total quantity of the standardized tincture of digitalis required to produce digitalization in an individual in India is more than double the amount

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required in temperate climates   On account of climatic conditions digitalis preparations undergo chemical changes, the content of therapeutically active glucosides being thus reduced and frequently toxic substances, the nature of which is not known, are formed   The result of this is that although in fresh tinctures there is a distinct relationship between the slowing of the pulse, the death interval and the M L D (minimum lethal dose) in the deteriorated and toxic tinctures this does not hold good   The M L D in these latter may or may not bear relationship to its therapeutic activity, but the change at once becomes apparent in the heart-beat, i e , there may be no slowing at all or the heart may stop suddenly due to the toxic effect of the drug when given by the intravenous route   Further we have shown that, by our method, one cat often gives sufficient information regarding the strength of a preparation and it is only necessary to use more animals in a few doubtful cases where slowing of the beat and M L D do not correspond

In this method the drug is diluted with normal saline and injected intravenously into anæsthetized cats   The lethal dose the death interval and the slowing of the heart-beat observed with a stethoscope are recorded   The slowing of the beat (therapeutic effect) produced by digitalis glucosides on a cat's heart is proportional, in case of standard tinctures, to the percentage of the active glucoside present, and the M L D , with due regard to the death interval   These observations may be graphically represented in the form of an equilateral triangle —

M L D

Death   Pulse
interval   rate
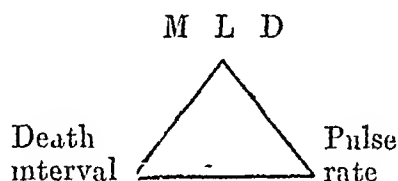
When tinctures become deteriorated or toxic, the relationship between the three is disturbed and shows marked variations and from these variations the potency and toxicity of a tincture can be worked out

In the British Pharmacopœia (1932) changes have been introduced in the standards and in the methods of biological standardization   We have, therefore, thought it necessary to revise the working standard in our laboratory with due regard to the altered conditions   With this object in view, we tested the standard digitalis powder, supplied to us through the courtesy of Sir Henry Dale of the National Institute for Medical Research, London, on a series of cats   The powder is stored in hermetically sealed tubes at a temperature below $0°C$ The extract was prepared according to the instructions laid down in the British Pharmacopœia (1932) by percolating 1 0 g of the standard powder with 25 c c of dehydrated alcohol   The extract so obtained was concentrated to a volume of 5 c c and then diluted with distilled water up to 10 c c , the diluted extract represents 1 g by weight of the standard digitalis powder   One g of the British standard powder represents 11 6 international cat units and as 1 g is present in 10 c c of the extract, 1 international cat unit is present in 0 086 g   This means that 0 86 c c of the solution (containing 0 086 g of powdered leaf) should kill a cat weighing 1 kilogram in one hour   The extract so prepared was kept in cold storage and every time was taken and diluted up to 20 c c with normal saline only

Method of assay —A series of cats weighing between 1 5 and 2 8 kilograms was selected Before biological assay they were starved for 24 hours and then anæsthetized with chloralose (0 1 g per kilo fed through a stomach tube) When the animal is under complete anæsthesia, which usually occurs within 2 to 2½ hours, it is fixed on a table and a glass cannula is fixed into one of its femoral veins and the digitalis solution is allowed to flow slowly and continuously into the vein at the rate of about 2 c c in 5 minutes per kilo of body-weight The rate of flow is so arranged that 20 c c of the diluted solution is administered in about 50 minutes This amount should be approximately a minimal lethal dose per kilo of cat and usually stops the heart in the above time The flow can be more accurately regulated by passing a long capillary tube into the burette and in addition to the stop-cock a screw clamp is fixed on the rubber tubing connecting the burette to the glass cannula This enables the flow to be regulated to any speed, however small

Working standards —A perusal of Table I will show the M L D per kilo in 14 cats with the standard digitalis powder The slowing of the heart-beat was recorded along with the death interval in every animal with a view to establishing standards for this country The range of deviation within which one may work was also fixed by the formula of van Wijngaarden 6 67 $\sqrt{n-1}$ The male and female cats have been grouped separately and the range of variation of M L D and the slowing of beat recorded The averages observed in Table I are —

1 Average lethal dose per kilo—17 57 c c (dilution 1 in 20)
2 ,, ,, ,, ,, ,, of standard digitalis folia—0 0879 g
3 ,, maximum slowing—35 3 beats
4 Range of maximum slowing—22 per cent of the initial rate

These figures will be used by us in future as standards for comparison in this laboratory

DISCUSSION

1 *Artificial respiration* —The British Pharmacopœia requires that artificial respiration should be employed but in our assay work we have found it unnecessary A perusal of the tables will show that the results, obtained in a series of 14 cats in which artificial respiration was not employed (Table I), do not differ materially from a series of 10 cats (Table II) in which it was employed The slowing of the heart was undoubtedly more in the latter series and the lethal dose was also somewhat larger due to prolongation of the death interval, but this did not alter the conclusion which could be drawn The M L D in the series, without artificial respiration, came closer to the League of Nation's standard Our conclusions in this respect are in accord with those of Smith and McClosky (1925) who found that the employment of artificial respiration renders the determination of the end-point more difficult and results more irregular

2 *Sex* —The female cats vary much more in their response to digitalis than male cats This is probably due to the fact that they are weakened by pregnancy and lactation It is, therefore, advisable to use male cats whenever possible and to exclude lactating and pregnant females

TABLE I

Biological assay of standard digitalis powder with cat method without artifical respiration

Number	Sex and character	Total weight, g	Death Interval		Heart rate in minutes Maximum slowing	Percentage of slowing	M L D per kilo, c c	Digitalis equivalent in g	Percentage of dose giving maximum slowing
			Hour	Minutes					
1	Male, white	2,230	1	5	28	18 9	22 4	0 112	75 0
2	Male, white	1,870	0	45	40	30 7	19 2	0 096	44 0
3	Male, white	2,190	0	40	36	19 3	14 6	0 073	62 5
4	Male, white	1,685	0	55	48	26 6	17 8	0 089	50 0
5	Male, white	1,310	0	45	6	3 3	17 1	0 086	55 5
6	Male, white brown	2,500	1	10	25	22 1	24 0	0 12	66 6
7	Female	2,990	1	30	68	34 7	19 0	0 095	52 6
8	Female, white	2,188	1	0	44	30 5	18 3	0 0915	50 0
9	Female, black and white	2,235	0	50	28	21 6	16 0	0 08	44 4
10	Female, black, white and brown patches	2,590	1	5	36	21 6	18 5	0 1035	50 0
11	Female, white, tiger spots	1,740	1	0	36	25 7	20 6	0 103	33 3
12	Female, white	1,540	0	40	12	8 3	13 6	0 068	42 8
13	Male, white with black	2,240	0	55	61	29 3	17 4	0 083	61 5
14	Female, white with brown	1,515	0	45	26	16 0	17 5	0 0875	33 3
AVERAGES			0	56	22 0	35 3	17 57	0 0879	51 5

TABLE II

Biological assay of standard digitalis powder with cut method with artificial respiration

Number	Sex and character	Total weight, g	Death interval Hour	Death interval Minutes	Heart rate in minutes Maximum slowing	Percentage of slowing	M L D per kilo, cc	Digitalis equivalent in g	Percentage of dose giving maximum slowing
1	Male, black and white spots	2,570	1	35	26	20	18 0	0 09	38 8
2	Male, white and brown	2,880	1	0	44	31 4	23 6	0 118	70 6
3	Male, black and white	2,820	0	40	42	30 0	16 3	0 0815	43 5
4	Male, white and brown patches	1,600	1	2	40	20 4	20 6	0 103	36 3
5	Male, white with few black patches	2,540	1	0	10	30 3	21 6	0 108	54 5
6	Female, black and white	2,205	1	20	54	38 7	27 2	0 136	33 3
7	Female	1,970	1	25	35	30 0	23 7	0 118	57 1
8	Female, grizzly white	1,630	0	55	80	40 0	22 2	0 101	45 4
9		1,935	1	15	61	34 6	22 7	0 113	40 9
10		1,345	0	40	48	28 0	14 8	0 0743	50 0
	AVERAGES		1	6	47	33 3	20 87	0 1043	47 9

3   *Slowing of the pulse rate* —The scientific interpretation of the action of a cardiac tonic is best explained with the help of an electrocardiograph   Its action on the origin and conduction of impulses, facilitating the passage of the contraction wave through the A-V bundle and its effect on the tone of the cardiac muscle can be studied with this instrument   These effects are manifested clinically by the increase or decrease of the pulse rate   Robinson and Wilson (1918) by electrocardiographic experiments on cats, have shown that there is a delay in the conduction of the A-V bundle impulses when 50 per cent of the M L D has been administered, and there is a lengthening of the P-R interval as well if the vagi are intact   If, on the other hand, the vagi are cut the T-wave is also inverted after 30 per cent of the lethal dose has been given   After 70 to 80 per cent of the M L D is given, idioventricular changes occur and there is a constant dissociation of the A-V wave   The slowing of the pulse rate is maximum after 25 per cent of the total dose   Levine and Cunningham (1920) by electrocardiographic studies found that the average margin of safety (i e , the difference between the minimum lethal dose and minimum toxic dose) was 60 per cent   For these reasons we have adhered to our method of recording the slowing of the heart-beat along with the M L D and the death interval   Tables I and II show that with standard tincture we may expect the maximum slowing of the pulse rate between 25 to 60 beats per minute   Our observations agree with Robinson and Wilson, and Levine and Cunningham, that a dose between 30 and 50 per cent of the M L D produces a maximum slowing of the heart rate and, after 80 per cent, the heart-beats become so irregular that it becomes impossible to differentiate individual beats by palpation or auscultation

4   *M L D* —With a dilution of 1 in 20 of the standard digitalis preparation, an average dose of 17 5 c c will be required to kill a cat of one kilogram in about one hour   The British standard of digitalis powder, when compared with International standard, shows one cat unit requires 0 0862 g of digitalis powder to kill a cat of one kilogram , in Table I our digitalis equivalent (column 8) comes to 0 0879 g This figure approaches closely the International standard, hence our method of assay appears to give a correct interpretation

5   *Death interval* —The League of Nations has fixed the duration of the experiment to about 40 minutes (varying from 30 to 55 minutes)   In our series the average death interval was about 56 minutes (it ranged between 40 and 90 minutes)   This variation may be due to the fact that we kept the *rate of* infusion of the extract at 2 c c in 5 minutes so that about 20 c c could be administered in 50 minutes   The rate of administration was kept constant in every animal *so that* the death interval depended upon the weight and the resistance of the individual to digitalis glucoside

6   *Range of deviation* —Table I shows that in the series of 14 cats the range of individual deviation according to van Wijngaarden formula (6 67 $\sqrt{n-1}$) comes to 24 01

7   *Number of cats required for an assay* —We have graphically represented the three important items to which we attach very great importance in the form of an equilateral triangle   If the two sides of the triangle come within the range and the third one varies slightly a favourable opinion may be given   If, however, a marked difference in the figure is obtained, one or more animals should be employed for assay and the mean of the results taken

Summary and conclusions

(1) Chopra's modification of Hatcher and Brody's cat method has given uniformly good results in the assay of digitalis preparations in India   It enables one to determine the strength of a preparation as well as any toxic changes produced in the glucosides as a result of climatic conditions

(2) The average M L D per kilogram of cat, worked in a series of 14 cats, approximates closely to the International standard, i e , 0 0879 g as compared with 0 0862 g

(3) It is not necessary to use artificial respiration   Its employment renders the end-point more difficult to determine and the final results are more irregular

(4) Records of the changes in the heart-beat give valuable information regarding the therapeutic efficacy of a preparation in the Tropics

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BIOLOGICAL ASSAY OF DIGITALIS PREPARATIONS IN THE TROPICS

Part V.

POTENCY OF *LANADIGIN* (GLUCOSIDE OF *D LANATA*) AND ITS RELATION TO THE STANDARD DIGITALIS POWDER (B P 1932)

BY

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THE spectacular effects of digitalization in cardiac insufficiencies have long been noted by clinicians but cases have been recorded where the use of digitalis preparations has been attended with toxic cumulative effects   Cases have also been met with where ordinary digitalis preparations could not be administered by the mouth on account of the presence of gastro-intestinal disturbances   Besides this, most of the digitalis preparations in ordinary use are liable to deteriorate on storage and in unfavourable climatic conditions and thus produce irregular results   Attempts have, therefore, been made by pharmaceutical chemists to isolate the active principles of the drug and present them in a stable form which would retain the therapeutic effects without the toxic manifestations   Such efforts have not been successful with *D purpurea* preparations but recently excellent results have been obtained with another variety

A number of species of digitalis have the same physiological action, though differing in their degree of potency   For instance *D purpurea* is more effective than *D campanulata* or *D alba* but *D ambigua*, from Austria, shows a therapeutic activity equal to *D purpurea*   *D lanata*, growing abundantly in the same country, has been shown to be therapeutically more active than *D purpurea*   Mannich (1933), Merz (1933) and Mohs (1933) succeeded in isolating four hitherto unknown glucosides from this plant among which *lanata* glucoside No 1 or *lanadigin* is of particular interest   This is a pure crystalline glucoside which has been reported to have little cumulative effect and can be washed out of the tissues much more easily than the glucosides of *D purpurea*   Pandigal, a preparation recently introduced by P Beiersdorf & Co , is a watery solution of the pure glucoside of *lanadigin* for injections and an alcoholic solution for oral use   Its advantage over other galenical preparations of digitalis is that it contains a single active principle in solution and is, therefore, uniform in strength and produces definite clinical effects There is also no likelihood of any side-effects being produced by saponins, tannins, chlorophyl and other soluble products which are usually present in the galenical preparations made from the leaf   *Lanadigin* produces a temporary dilatation of the peripheral blood vessels followed by a constriction   It contracts the kidney vessels but at the same time increases the output of urine   It has also been reported that it does not deteriorate rapidly and that solutions of *lanadigin* can be kept for 1½ years without any loss of potency   The other advantage claimed is that it can be sterilized by boiling   Dietrich and Schwiegk (1934) considered the introduction of this preparation a real advance in the employment of pure and active therapeutic substances

Stoll and Kreis (1933) showed that *D lanata* contains three different glucosides A, B and C, though separation is not easy since all of them are amorphous and form a homogeneous crystalline mixture with each other These workers reported that the crystallized glucosides of digitalis are not originally present in the crude drug as such but are produced in the process of enzyme disintegration by the separation of the sugar molecule from the glucoside which is originally present in the plant   Smith isolated a new glucoside called *digoxin* from *D lanata*   It is a colourless, crystalline substance with a melting point at 265°C , its structural formula is $C_4H_{64}O_{14}$, it is soluble in alcohol but is insoluble in water, chloroform, ethyl acetate and acetone   It is optically active and hydrolyses into a digitose and a crystalline genine   The three different glucosides have now been put on the market in the crystalline form as *Digilanide* (Sandoz & Co) or as *Digoxin* (Burroughs Wellcome & Co) but *lanadigin* and its preparations have been more extensively used

Clinical uses —*Lanadigin* has been highly recommended since it can be administered by the mouth, *per rectum* or intravenously with equally good results   When administered by mouth it is absorbed rapidly, producing no nausea or vomiting   Its use *per rectum* has been advocated in advanced cases of cardiac dilatation with ascites   In such conditions the glucoside, if administered by mouth, is not absorbed and does not reach the general circulation   If administered *per rectum*, however, it is absorbed readily by the rectal mucosa and is carried directly to the heart via the inferior hæmorrhoidal veins and

inferior vena cava instead of passing through the mesenteric vessels and the
engorged liver In very advanced and urgent cases it can be administered
intravenously twice daily in doses of 0 4 mg to 0 6 mg dissolved in 10 c c
of physiological saline solution Its advantage over digitoxin and strophanthin
is, therefore, obvious Clinical trials in patients with auricular fibrillation
by E J Wayne of the Therapeutic Trial Committee showed that it reduces
the ventricular rate rapidly and removes œdema and congestion Usually
0 5 mg of *lanadigin* is dissolved in 1 c c of 80 per cent alcohol and when
further diluted with 9 c c of normal saline solution the percentage of alcohol in
solution is reduced to 8 per cent This concentration of alcohol is not harmful
when injected intravenously Digoxin has been recommended in auricular
fibrillation Its initial dose by mouth is 1 mg to 1 5 mg followed by 0 25 mg
every 6 hours , intravenously it is given in 0 75 mg to 1 0 mg doses, the
drug being continued till the pulse rate falls to 60 to 70 beats per minute
The desired slowing is obtained rapidly whether the drug is given by mouth
or intravenously

A perusal of what has been said shows that the introduction of the prepara-
tions of *D lanata* is a useful advance in cardiac therapeutics We, therefore, carried
out a study of the physiological potency of these glucosides as compared with
D purpurea and also investigated whether other claims made regarding them were
justified We used the preparation *Pandigal* as well as the pure glucoside in these
studies and found them equally effective

Biological assay —According to Hempshire (1933) the leaves of *D lanata* are
about three to four times more potent than the international digitalis powder One
gramme of *lanadigin* represents 0 3 gramme of digitalis leaf, in other words it is
about one-third as toxic as the latter , 0 31 mg of *lanadigin* has been reported to be
one cat unit (Hatcher and Magnus method) Merz (*loc cit*) reported that the exact
frog unit with *lanadigin* is difficult to determine One gramme, however,
represents 450,000 frog units (Houghton and Straub) One milligram of digoxin
as measured by the frog method has been found to be equivalent to 0 28 mg of
standard ouabain

It has been stated above that *Pandigal* can be easily standardized as it is a
solution of a single glucoside, *lanadigin* only To do this *lanadigin* was first
dissolved in a few drops of absolute alcohol and further diluted with water
before use

The watery solutions of *lanadigin* thus prepared were assayed in a series of 10
cats with the object of comparing its effects on the heart rate, death interval and
the M L D as compared with the national standard digitalis powder (B P 1932)
It has been reported that 0 31 mg of *lanadigin* is lethal per kilo of cat when injected
intravenously Three mg of fresh glucoside were dissolved in about 1 c c of
absolute alcohol and then diluted with 10 c c of normal saline One c c of this
solution, therefore, represented 0 3 mg of *lanadigin* which is alleged to be the
minimum lethal dose per kilo of cat One c c of this solution (0 3 mg *lanadigin*)
was further diluted with 10 c c of normal saline and was administered intravenously
to cats under chloralose anæsthesia at the rate of 1 c c per kilo every five minutes
according to the procedure laid down in Chopra's modification of Hatcher's method

(1933) This administration was continued till the animal died The results obtained are tabulated in Table I —

TABLE I

Giving the results of assay of lanadigin *in 10 cats by Chopra's method*

Number	Sex and character	Total weight, g	Death Interval		Maximum slowing in heart rate	Amount of lanadigin per kilo cat in mg
			Hour	Minutes		
1	Female, white spots	1,930	0	55	32/120	0 342
2	Male, black	2,960	1	10	42/176	0 423
3	Male, black and white	2,680	0	30	32/198	0 168
4	Male, white	2,680	0	30	20/198	0 168
5	Male, white	1,712	0	35	12/172	0 21
6	Male, white .	2,420	0	35	54/240	0 2169
7	Male, brown and white	3,080	0	40	54/224	0 234
8	Female, black and white	1,615	0	45	6/156	0 2505
9	Female, white	1,500	0	45	54/210	0 270
10	Male	2,730	1	5	48/204	0 3294
	Averages		0	45	35 4	0 26118

In Table II are the averages from the results obtained in Table I, and the average results obtained by us (Chopra *et al* , 1934) with the international digitalis powder and kept as a standard in our laboratory

A perusal of Table I will show that the cats died in about 45 minutes, the average slowing was 35 beats per minute and the M L D is 0 26118 mg of *lanadigin* per kilo   When compared with our laboratory standard of international digitalis powder it will be noted that with *lanadigin* (Table II) the animal died in comparatively shorter time, but the average slowing of the pulse rate was the same   The M L D for cats as noted above is 0 26118 mg of *lanadigin*, while for the standard digitalis powder it is 0 0879 g or 0 2437 mg of digitoxin   The digitoxin content of *Digitalis purpurea* leaf varies from 0 1 to 0 4 per cent   Taking 0 3 per cent for calculation (Solis-Cohen and Githens, 1928) we obtain the above figure The toxicity of *lanadigin*, as compared to the glucosidal content of *D purpurea* leaf, therefore appears to be the same   *Lanadigin* produces an equal amount of slowing of the pulse rate in a comparatively shorter time than the standard digitalis powder

As regards the keeping properties of *lanadigin* in tropical climates these are under investigation   From certain observations we have already made there appears to be no doubt that this glucoside and its solution *Pandigal* are much more stable in a climate such as that of Calcutta than *D purpurea* preparations   There is also no doubt that *lanadigin* can be boiled without decomposition and is easier to administer effectively by the intravenous route

SUMMARY AND CONCLUSIONS

1   *Lanadigin*, a glucoside occurring in *D lanata*, is physiologically as potent in animals as the glucosides of *D purpurea*   In cats it has practically the same power of slowing the heart rate and is lethal to the animals in practically the same time

2   *Lanadigin* can be sterilized by boiling without decomposition and does not deteriorate as rapidly under tropical conditions as the glucosides of *D purpurea*

3   The therapeutic effects can be obtained more rapidly and with less inconvenience to the patient

4   *Pandigal* (Beiersdorf), a solution of this glucoside, is a stable and therapeutically active preparation

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SOME INORGANIC PREPARATIONS OF INDIAN INDIGENOUS MEDICINE

Part I

ABHRA BHASMA

BY

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THE great majority of preparations found in the materia medica, both of Hindu and Mahommedan medicines in India, is of vegetable origin and the number of inorganic preparations is very small   It is well known that one of the earliest works on Hindu medicine by Charaka does not deal at all with any mineral drugs and Susruta, written at a later period, only mentions the use of a few natural salts   It thus appears that the art of adopting the metals and metallic compounds for medicinal purposes was not highly developed among the ancient Hindus   Only writers of considerably later periods gave descriptions of calcination and purification of compounds and of processes of converting metals into suitable forms for use as medicaments   Mahommedan physicians also made use of inorganic preparations to a very limited extent

One group of such inorganic preparations was made from certain metals or from some crude ores by the process of repeated heating to a high temperature and then cooling, generally after mixing them with certain organic substances of animal or vegetable origin   The resulting products are various mixtures of metallic particles in a fine state of division or carbonates or oxides of different metals together

with various proportions of silica   The mass thus produced is finely ground and has the appearance of a buff or grey powder   Such preparations are known in Ayurvedic medicine as 'Bhasma' and in Tibbi medicine as 'Kushta', both implying that the metal in the preparation is destroyed and is converted into ash-like substance

All sorts of remarkable properties are attributed to these compounds and they are very largely used by practitioners of the Ayurvedic and Tibbi systems of medicine in India   It was, therefore, considered desirable to study these preparations by analysing them and working out their pharmacological action and therapeutic properties   In this paper we propose to give the result of our investigation with the first of these preparations we have studied, i e , *Abhra Bhasma* or the ash of mica

I

'*Abhra Bhasma*'

or

The Ash of Mica

Abhra, Abh or mica is a mineral which occurs in sheets of moderate thickness forming a compact mass and capable of being cleaved into flexible plates of extreme thinness   It is widely distributed in India, the principal source of supply being the districts of Hazaribagh, Kodarma, Jamtara, Gaya, Monghyr, some parts of Rajputana and the Punjab and some granite veins in the hills of Mysore and the Western Ghats   The chief constituents of mica are potassium, aluminium, silica, magnesium and iron with traces of lime   Four principal varieties are mentioned by Hindu medical writers, namely, white, yellow, red and black , only the last-mentioned variety, which is the mineral Biotite, $K_2H Al_3 (SiO_4)_3 (Mg, Fe)_6, (SiO_4)_3$, is used for medicinal purposes

The black variety, again, is classified under four distinct heads according to certain physical properties   When thrown into fire, 'Dardur' (frog) leaps like a frog in the fire (due to explosions)   'Nag' (snake) produces a hissing noise like that of serpents , 'Pinak' (the bow or trident) separates into layers on the fire , and the last but most important variety, known as '*Vajra abhra*' (impenetrable like thunder) is so called because it remains quiet even on strongly heating When administered, the black 'Dardur' is believed to cause death, the 'Nag' leprosy, the 'Pinak' fistula, but the last variety, when properly purified, is not injurious to the human system   Consequently, it is only the *Vajra abhra* which is used in Ayurvedic medicine

Purification —The black *Vajra* variety of '*abhra*' is burnt in fire made of cowdung cake and, while red-hot, is dipped into pure cow's milk   Its layers are then separated and soaked in the juice of *Amaranthus polygamus*, a kind of vegetable commonly known as *Kanta notay* in Bengal, together with some acid, preferably Kanji (vinegar), for eight days   It is then known as 'Sodhita' or purified *abhra* This is mixed with one-fourth of its weight of Shali Dhanya (a variety of paddy) tied in a blanket and soaked with water for three days   The *abhra* contained in the blanket is then rubbed by hand, when fine sandy particles pass through the interstices of the fabric and are collected for use   This is known as 'Dhanyabhra'   It

is further treated with cow's urine and rubbed in a mortar and the pasty mass is heated by a process known as 'Gajaputa' which is done by putting the paste in a closed crucible consisting of two concave earthen basins placed one above the other and the joints closed by a mixture of cowdung with earth   It is then subjected to a very strong heat   The '*abhra*' loses all its shining particles and acquires a brick-red colour   This is known as '*Abhra bhasma*'   Sometimes the ignition is repeated several hundred times and the efficacy of the medicine is said to be enhanced by the number of such ignitions   When ignited one thousand times it is known as '*Sahasraputita Abhra*'   It is of a buff colour and has a slightly saline and earthy taste

'*Abhra bhasma*' undergoes another process of purification known as '*Amrit-karan*' or nectarification   Two seers of the decoction of *Trifala* or three myro-balans consisting of *Phyllanthus emblica, Terminalia chebula* and *Terminalia bellerica* together with one seer of clarified butter, and one-fourth seer of *Abhra bhasma* are mixed together and heated in an iron pan at a low heat till the mixture dries up   It is then powdered and used as such   Besides these there are other processes of purification of *abhra*

At the suggestion of M M Kaviraj Gananath Sen, we analysed a sample of '*Abhra bhasma*' supplied to us by the Kalpataru Ayurvedic Works of Calcutta   It was a buff coloured amorphous powder with a very slight saline taste   On analysis one hundred parts of the sample was found to contain the following ingredients —

		Percentage
Silica	SiO_2	36 01
Ferric oxide	Fe_2O_3	12 78
Alumina	Al_2O_3	27 57
Lime	CaO	5 03
Magnesia	MgO	1 92
Potash	K_2O	13 17
Soda	Na_2O	3 06
Chlorides	$NaCl$	0 09
Sulphates		*Nil*
Phosphates		Very faint trace
Nitrates		*Nil*
Moisture		0 37
Total		100 00

We then investigated the total water-soluble portion and found that 6 666 parts were soluble in boiling water   The soluble matter consisted of —

		Parts
Silica	SiO_2	2 094
Iron and alumina	Fe_2O_3 and Al_2O_3	0 055
Lime	CaO	0 192
Magnesia	MgO	1 118
Potash	K_2O	2 924
Soda	Na_2O	0 196
Chlorides	$NaCl$	0 087
Total		6 666 parts

Lastly, we digested 2 5 grammes of the sample in 250 c c of 0 26 per cent hydrochloric acid solution, the approximate strength of acid found in gastric juice, at a temperature of about 37°C for twenty-four hours   The total solubility was 31 288 parts which on analysis gave the following results :—

		Parts
Silica	SiO_2	6 645
Alumina	Al_2O_3	8 300
Iron oxide	Fe_2O_3	2 116
Magnesia	MgO	1 907
Lime	CaO	0 884
Potash	K_2O	8 377
Soda	Na_2O	3 059
	TOTAL	31 288 parts

From the results of the last two analyses it appears that the various preliminary treatments, known as ' purification ', have altered some of the properties of the mineral   At the high temperature to which it is subjected it is hardly possible that any of the organic matter could have been left behind and the analyses bear out this assumption   The treatments have possibly converted a portion of the mineral into oxides or carbonates or into some other form which can be dissolved out more easily   It was originally almost insoluble in water or in dilute hydrochloric acid but, after the treatment, a high percentage goes into solution, especially in acid of approximately the strength found in gastric juice

Pharmacological action and therapeutic uses—*Abhra bhasma* is considered to be a tonic and, in combination with preparations of iron, it is used in chronic diseases such as diarrhœa, dysentery, fever, diabetes, anæmia, jaundice, enlargement of spleen, etc   Its efficacy is said to be increased when given with iron   The drug is usually prescribed in doses of 6 to 12 grains

The results of analysis given above show that a high percentage of the metallic constituents exist in a soluble form and the pharmacological actions and therapeutic properties of some of these are known   Whether these produce any remarkable effects in the dosage in which they are prescribed by indigenous practitioners is problematical for they are never given alone but always in combination with various preparations of organic and inorganic origin   We have tried this preparation by itself in a number of patients suffering from diabetes without producing any apparent effects on the urine or blood-sugar   Small doses of metallic substances absorbed may produce stimulation of the tissues generally and hæmaturic effects, but these were not very remarkable in our series of cases and it was not considered worth while to proceed with further trials

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SOME EXPERIMENTAL OBSERVATIONS ON THE VENOM OF THE INDIAN COBRA

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THE venom of the Indian cobra, *Naia naia vel tripudians,* has been the subject of extensive researches during the past and its action has been studied in great detail by numerous investigators with a view either to discover an effective antidote to it or to inquire into the possibilities of its use in therapeutics   The observations that have been recorded by different workers on the symptoms of poisoning due to the venom and the changes produced by it on the various organs are more or less similar in essential details   The consensus of opinion is that in the poisoning, failure of respiration is the cause of death, that circulation survives for some time after the breathing ceases and that in most cases the heart continues to beat for hours if the aeration of the blood is maintained by artificial respiration

The question, however, whether the respiratory paralysis produced by the venom is due to central or peripheral action is still open as the observations made by previous workers on this point are of a conflicting nature   Aron (1883), working on frogs, found that the peripheral nerve-ends were not paralysed and that death was due to the paralysis of the spinal cord only   Wall (1883) observed that 0 5 c c of the fresh venom injected into frogs exerted a weakening effect on the peripheral motor nerves accompanied by a similar effect on the central nervous system, especially the spinal cord   Ragotzi (1890) came to the conclusion that the characteristic action of cobra venom in both frogs and rabbits was on account of the paralysis of the motor nerve-ends and stated that Aron got different results because he had not used a sufficiently small dose of the venom   Wall's experimental results were also subject to a similar error   Vollmer (1893) maintained that the central nervous system was also directly paralysed besides the motor nerve-ends   Rogers (1903) and Elliot (1905) thought that failure of respiration due to paralysis of the centre was the cause of death in cases of cobra venom poisoning   Cushny and

(289)

Yagi (1918), on the other hand, asserted that the respiratory failure was the result of peripheral paralysis and that the central nervous system was not directly involved in the action   As recently as last year, Kellaway (1933) during a discussion on ' Some peculiarities of Australian snake venom ' upheld that the action of snake venoms on the respiratory centre was peripheral and not central

' ' Working with the gastrocnemius muscle-nerve preparations of frogs, we found that with the nerve alone immersed in a 1 in 20,000 solution of cobra venom in Ringer's solution, the excitability of the nerve to electrical stimulation after the lapse of ten minutes was not in any way different from that of the control nerve kept in pure Ringer's solution for a similar period   Whereas, if the muscle alone was dipped into the solution of the venom, its irritability to electrical stimulation through the nerve, which was greater than the control for a short period at the beginning, gradually became weaker and finally disappeared altogether   At this stage the muscle responded to direct stimulation for a short time, after which, even this stimulation had no effect   Excitability to direct stimulation was lost earlier if the venom had been injected into the muscle

The diaphragm of a cat anæsthetized with chloralose was exposed on the left side of the abdomen as also the phrenic nerve of the same side   The diaphragm was hooked from the abdominal side and connected to a lever through a system of pulleys   The phrenic nerve was stimulated with a minimal tetanizing current every ten minutes for a period of forty minutes before and after the injection of 0 05 mg of the dried venom into the femoral vein   It was found that whereas the response of the diaphragm to stimulation through the nerve was constant before the injection, yet, after the injection the diaphragm gradually failed to react to the stimulation (Graph 1)   Long after the muscle had ceased to be irritable through the nerve, direct stimulation of the muscle was found to produce strong contractions

Thus, while the irritability of the normal muscle to the tetanizing current through the nerve remains constant, the muscle acted on by the venom, is found, however, gradually to fail to respond to a similar stimulation   This, considered together with the observation that the nerve whether subjected to the action of the venom or not, induces contraction of the connected muscle when stimulated, seems to indicate that the end-plates block the transmission of the stimulus to the muscle   But to produce this phenomenon very small quantities of the venom have to be used and allowed to act for a long period   With bigger doses animals die long before the paralysis of the end-plates develops and this is probably the reason why, with lethal doses of the venom, the stimulation of the phrenic nerve immediately after death is found to give rise to contractions of the diaphragm in the experimental animal

The symptoms manifested in cases of cobra bites also afford evidence of paralysis of the end-plates   In about half the number of fatalities, where death occurs in about seven hours after the bite, the integrity of the central nervous system is maintained long after the patient finds it impossible to use many of the muscles of his body   In these cases, apart from the pain caused by the bite, the victim develops symptoms such as the following   Loss of control over the muscles of the legs, staggering, falling of the lower jaw with oozing of frothy viscid saliva from the mouth, indistinct speech, moaning, shaking of the head from side to side and

quickened respiration   Forty minutes afterwards, the patient is unable to answer questions but appears conscious and his arms are not paralysed   The respirations are laboured, though not stertorous, and gradually become slower and cease without convulsions   Loss of control over the muscles of the legs, jaw, tongue and larynx are typical of peripheral paralysis and the order in which weakness of the muscles is felt seems to indicate that the muscles that are used most frequently get fatigued earlier   While locomotion and speech are impossible and respiration is getting feeble, the patient is able to use his arms and his consciousness fails only towards the end   These symptoms show that paralysis of the central nervous system follows that of the nerve-ends   But in about ten per cent of the cases where the victim dies within an hour of the bite, cardiac failure sets in too early for the manifestation of the signs of paralysis of the end-plates and death, in these cases, is due to the affection of the central nervous system, respiration failing first as the result of inadequate circulation and deficiency of oxygen   If the dose of the venom injected by the cobra at the time of the bite is sublethal, the paralysis of the end-plates that develops gradually passes off and in time the patient recovers   Hence it appears that though cobra venom, in common with the other snake venoms, brings about paralysis of the nerve-endings, yet, death cannot be due merely to this cause because the quantity of the venom required to produce this effect alone would be far below the lethal dose   The majority of deaths on account of cobra bite may, therefore, be considered as generally due to the paralysis of the respiratory centre

While opinion is unanimous that with lethal doses of the venom animals always die of respiratory failure before the stoppage of circulation and the manifestation of any sign of paralysis of end-plates of the skeletal muscles, it will only be of academic interest to attempt to find out to what extent the effect of the venom on the nerve-endings is responsible for the respiratory symptoms, especially when it has been found that the antidotes against curare are not effective in cases of poisoning with cobra venom (Cushny and Yagi, *loc cit*)   What will be of greater importance from a practical point of view is the determination of the action of the venom in sublethal doses, as this is likely to afford valuable clues relating to suitable methods of treatment and to the possibilities of utilizing the venom in minute doses for therapeutic purposes as has been done with other known poisons like strychnine, aconitine, etc

But with regard to the effect of sublethal doses of the venom, the data published by various workers differ from one another to a great extent   While one has used 2 mg of the dried venom per kilo weight of a cat intravenously and was able to keep the animal alive for nearly an hour (Cushny and Yagi, *loc cit*), another has administered 0 2 mg per kilo weight to a cat by the same method and has found that the blood-pressure dropped down to the zero level within a few minutes (Chopra and Iswariah, 1931)   Again, when 0 8 mg of the venom produced on a cat weighing 2 52 kilos a persistent rise of blood-pressure, 0 6 mg administered in the same way to a cat, 3 38 kilos in weight, reduced the blood-pressure to zero (Chopra and Iswariah, *loc cit*)

Two samples of the fresh dried venom when injected in 0 1 mg doses into the femoral vein of cats anæsthetized with chloralose produced a marked and persistent rise of blood-pressure (Graph 2)   The same effect could not, however, be obtained with some other samples experimented with

To see whether this rise in blood-pressure was accompanied by increased output of the heart we perfused amphibian and mammalian hearts with fluids containing the venom in concentrations ranging from 1 in 10,000,000 to 1 in 5,000,000 and found that with the increased stimulation of the heart there was progressive decrease in the amount of outflow until at last the aortic pressure reached the zero level. This was apparently the result of the capacities of the chambers of the heart getting progressively reduced on account of the contractions of the muscles of the heart. The heart finally stopped in systole.

A frog's heart was made to cease beating by injecting a small dose of aconitine into the liver and 0 1 c c of a 1 in 10 000 solution of the venom was injected intra-hepatically thereafter. Immediately after the injection the heart revived, and a second dose containing the same quantity administered in the same way a few minutes later increased the tone and amplitude of the heart-beats still further (Graph 3). Though stimulation was evident with the second dose, the rate of the beats began to get slower and slower until at last the heart stopped in systole.

Involuntary muscles of the intestines and uterus also reacted to cobra venom by a similar increase in the tone (Graphs 4 and 5).

Yagi found that with the blood vessels of frogs and rabbits there were marked contractions of the vessels when the venom (0 2 c c of a 1 in 1,000 solution) was added to the Ringer's solution perfused through them and that the venom acted more peripherally than adrenalin on the muscle walls since the addition of ergotoxine to the perfusing fluid abolished the action of adrenalin on the vessels while leaving the action of the venom unchanged. The action of the venom, therefore, appears to be directly on the muscle substance itself resulting in the tonic contraction of the muscle.

When the blood-pressure was persistently high as in Graph 2, there were dips in the tracing. This was probably because the heart working to maintain such a high pressure for a long time got tired in consequence and missed a beat occasionally. If the dose of the venom administered was small and the aeration of the blood satisfactory the blood-pressure gradually returned to the normal after some time. In some cases after the blood-pressure had stood at a high level for a period the heart suddenly collapsed and the blood-pressure fell to zero. In these cases, if artificial respiration was given or if the quantity and force of the air pumped into the lungs were increased should the animals be already having artificial respiration, collapse of the heart was generally prevented (Graph 6). It will be seen from the graph that when the blood-pressure had dropped down, artificial respiration given immediately after has raised it. This shows that there is probably an increased resistance to the entry of air into the lungs as a result of which there is depletion of oxygen and accumulation of carbon dioxide in the blood, culminating in cardiac distress. The assumption that the increased resistance to the entry of air into the lungs is due to the tonic contraction of the involuntary muscles of the bronchi is supported by the observation that the venom induces a similar contraction of all other involuntary muscles.

Thus the muscles of the heart, intestines, uterus, blood vessels and bronchi all seem to react to cobra venom by tonic contraction.

The marked and persistent rise of blood-pressure appears, therefore, to be due to the combined effects of the tonic contraction of the heart muscles, of asphyxia

GRAPHS

Graph (1)

a_1 Before a_2 Before a_3 Cobra venom a_4 venom b_1 after b_2 cobra b_3 cobra venom b_4 Graph (1)

Time 5 Sec.

Graph (2)

Carotid pressure
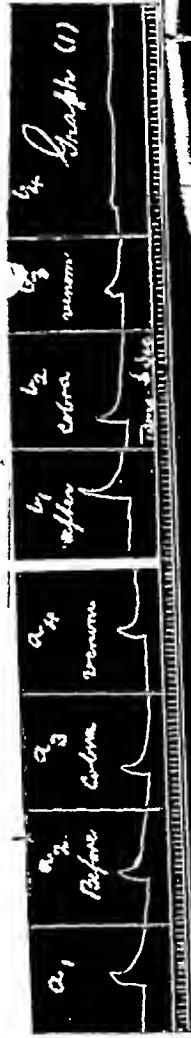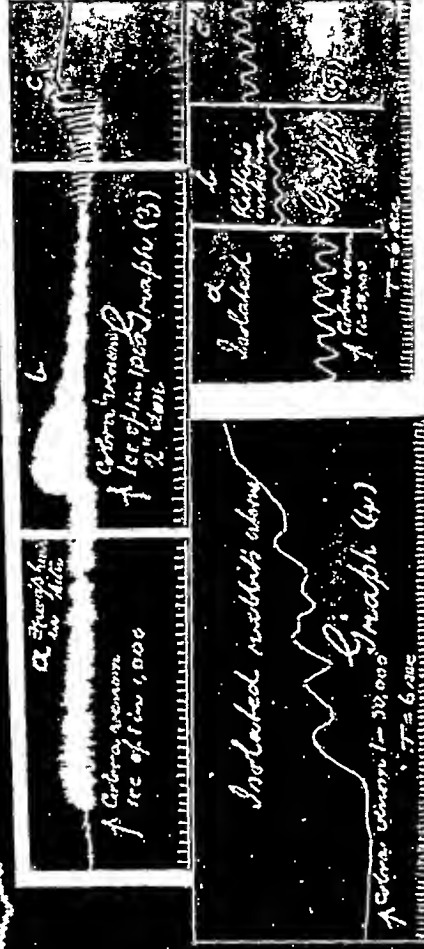
Cat 2 2 Kilos Chloralose

T= 6 seconds

Cobra Venom 0·1 mg

Graph (3)

a Frog's heart in situ

↑ Cobra venom 1 c c of 1 in 1,000

b ↑ Cobra venom ↑ 1 c c of 1 in 1,000 2nd dose

c

Isolated rabbit's uterus

↑ Cobra venom 1—50,000 T = 6 sec

Graph (4)

a Isolated b isolated c

↑ Cobra venom 1—50,000 T = 6 sec

Graph (5)

Carotid pressure

Decerebrated Cat

↑Cobra venom 0·1 mg (1)

Time = 6 seconds.

Graph (6)

(2)

Graph (6)

Graph 1 shows the effect of 0 05 mg of cobra venom on the contractions of the left diaphragm of achloralosed cat induced by stimulating the left phrenic nerve by a minimal tetanizing current, a_2, a, a_3 and a_4 show the contractions before and b_1, b, b_3 and b_4 after the administration of the venom Graph 2 Effect of 0 1 mg of cobra venom on the blood pressure of a chloralosed cat Graph 3 (a) Effect of a cobra venom (0 1 c e of a 1 in 10,000 solution) on a frog's heart that had stopped beating due to an injection of aconitine (b) Effect of a second dose of the venom on the same heart (c) Continuation of (b) after an interval of 84 seconds Time interval between (a) and (b) 200 seconds Note the stimulant effect of the venom in (a), increase of tone and amplitude in (b) and slowing followed by stoppage of the heart in systole in (c) Graph 4 Effect of cobra venom on the isolated uterus of a rabbit Concentration of the venom in the bath was 1 in 50,000 Graph 5 Effect of the venom on an isolated bit of cat's intestine Time interval between (a) and (b) 7 5 minutes and between (b) and (c) 10 minutes Graph 6 Record of blood pressure of a decerebrated cat under artificial respiration Venom was given at arrow mark (1), at arrow mark (2) blood pressure fell down nearly to zero level the volume and force of air pumped into the lungs were then increased Note the rise of

consequent on the narrowing of the lumen of the bronchi and of general vascular constriction

The cardiovascular effects produced by the administration of small quantities of cobra venom may be the reason for the use of the venom in ancient Indian medicine where it has been recommended for use as a cardiac tonic in extreme cases of heart failure   In typhoid, pneumonia, plague, cholera and epidemic diarrhœa, during the last stages, when every other medicine had been tried without benefit, it is said that cobra venom administered in very small doses in combination with mercury and sulphur is the only remedy that may be relied on to prevent collapse   Ghosh (1931) seems to have demonstrated the reviving properties of the venom by using carefully regulated doses in his experiments on plants

But in view of the observations that have been made to the effect that cobra venom is not absorbed through healthy mucous membrane, it is inconceivable that it can effect any beneficial changes by oral administration unless it be that the other ingredients with which the venom is mixed renders it absorbable as a result of some physical or chemical changes or so modifies the condition of the mucous membrane of the alimentary canal that the venom is directly absorbed from the gastro-intestinal tract   The mode of action is not explained in books on ancient Indian medicine and any claims as regards the alleged curative effects of the venom by administration by mouth have to be treated with reserve and caution in the absence of positive proofs in evidence thereof

On the strength of an observation that there was progressive local anæsthesia after cobra bite and on the assumption that this may be due to the affinity of the venom for certain phosphatide constituents of nerves, the venom has been used to alleviate the pain and to delay the progress of certain types of cancer   Monaelesser and Toquet (1933), who were the first to observe these effects, injected, at intervals varying from 3 to 15 days, doses of the venom ranging from 2 5 to 20 rat units, the rat unit being the minimum toxic dose for a rat weighing 20 g , and found that some of the patients suffering from cancer derived benefit from the treatment   Fitzsimons (1929) also claimed that the venom of the viper or adder in non-lethal doses is a cure for epilepsy, chorea and certain other functional disorders of the nervous system

But our observations on cobra venom go to show that its action is neither constant nor reliable and that the action differs a great deal between one sample and another and even between fresh and old samples of the same   The factors responsible for this difference are not definitely known and in the present state of uncertainty the indiscriminate use of the venom in therapeutics is likely to produce untoward results

Cushny and Yagi (*loc cit*) observed that, of two samples of cobra venom they had procured for their investigations, one was 10 times as powerful as the other   Calmette and Massor (1914) found that the venom lost its initial toxicity even when kept in a closed vessel protected from light   Our experimental findings are also of a similar nature   The intensity of action exhibited by a fresh sample of the venom was very much more than that of an old sample of the same, though the latter had been kept in a sealed glass-tube in a cool, dark place   The results obtained with old samples of the venom were similar in every respect to those published by Chopra and Iswariah (*loc cit*)   A corresponding deterioration was also noticed in regard to the toxicity of the venom on keeping   While the M L D of a fresh

specimen for cats intravenously was 0 075 mg per kilo of body-weight, the M L D of the same specimen after it had been kept for about a year was found to have changed to 0 5 mg per kilo of body-weight of the animal   If, therefore, the properties of the fresh venom observed with experimental animals are to be made use of for therapeutic purposes, the cause of its deterioration should be determined and steps should be taken to ensure that its potency and toxicity are within safe margins by devising suitable methods for its preservation and biological assay

As a result of the present study we are led to make the following observations The activity of cobra venom is a variable factor and our present knowledge concerning it is not sufficient to warrant its use as a therapeutic remedy   Further work on factors affecting its potency, stability and toxicity has to be done before it can be safely used in the treatment of disease

SUMMARY AND CONCLUSIONS

1   Lethal doses of the venom of the Indian cobra cause respiratory distress followed by death due to central respiratory paralysis

2   Sublethal doses of the venom so regulated as not to produce respiratory or cardiac embarrassment paralyse the motor end-plates alone some time after the administration of the venom

3   The venom, in small doses, produces in experimental animals a marked and persistent rise of blood-pressure   This rise is due to the combined effects of the venom in causing asphyxia, stimulation of the myocardium and general vascular constriction   Only certain samples of the venom and at some undertermined stage of their keeping were observed to produce the marked rise of blood-pressure

4   Injections of the venom in minute doses revive a heart that has been made to cease beating by the administration of aconitine

5   The venom produces tonic contraction of all involuntary muscles

6   There is a gradual diminution in the activity of the venom on keeping Therefore, if the cardiac effects displayed by the venom on experimental animals are to be made the basis of its rational use in therapeutics, or if the venom is to be used as a remedial measure on account of certain beneficial effects it has been mentioned to produce in the treatment of cancer, etc , by a few workers on the subject, it is imperative that methods should first be devised for the preservation of its potency and for its biological assay

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STUDIES ON THE ANTIGENIC STRUCTURE OF *VIBRIO CHOLERÆ*

Part VII

TWO ACID-SOLUBLE PROTEIN FRACTIONS

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IN the previous paper of this series we described the isolation and identification of two distinct proteins in the vibrio group, and outlined a classification of the smooth vibrios based on the presence of these proteins and of the two types of polysaccharide which we have studied earlier (Linton, Mitra and Shrivastava, 1934a) The two proteins, which we designated Protein I and Protein II, corresponded in large measure with the usual classification Protein I was found in vibrios derived from clinical cholera and Protein II in vibrios from water The variations from this scheme and their possible significance are now being studied

In our analyses of the two vibrio proteins by the van Slyke method we found that they had a very closely allied, if not identical, nitrogen distribution (Linton, Mitra and Shrivastava, 1934) We were, therefore, led to believe that the proteins of the two types might consist of the same units which were put together in two different ways The experiments reported in this paper will, we believe, strengthen this hypothesis

In this paper we are describing experiments designed to compare two of the fractions into which the proteins of the cholera and the cholera-like vibrios can be divided We shall take up in this report the chemical study of the acid-soluble proteins, and leave the question of the reaction of proteins in dilute alkali for a later study

As the table shows, the ' A ' and ' B ' substances occur in both the pseudo- and euglobulin portions of the vibrios, and appear to come in larger amount from the euglobulin fraction   This finding does not, of course, indicate anything about the position of the two acid-soluble fractions in the vibrio protein, since it is probable that the globulins represent simply fractions of varying solubility in the whole protein

Another series of experiments was carried out to determine whether keeping the vibrios in distilled water in the refrigerator would, by increasing the dispersion of the proteins, also increase the yields of ' A ' and ' B '   Two hundred and fifty bottles of a 48-hour growth of an El Tor vibrio (Protein II) were washed several times in distilled water in the Sharples supercentrifuge, and taken up in one litre of distilled water   Samples were taken at once and at intervals of 6, 14 and 16 days, and the amounts and percentages of ' A ' and ' B ' determined The results are set forth in Table III —

<div align="center">TABLE III</div>

Influence of distilled water on the yields of ' A ' and
' B ' substances

Interval.	Calculated weight of vibrios, g	Weight of ' A ', g	Percentage of ' A '	Weight of ' B ', g	Percentage of ' B '
At once	1 33	0 02	1 5	0 06	4 5
6 days	1 36	0 04	3 0	0 07	5 1
14 „	1 27	0 06	4 7	0 06	4 7
16 „	30 36	1 28	4 2	1 64	5 4

The acid-soluble ' A ' substance appears to increase under the conditions of the experiment   If this increase is real it may point to the gradual formation of ' A ' from protein in distilled water, since as we have already pointed out, ' A ' is readily separated from the vibrios, and practically the maximum yield is obtainable by one extraction   ' B ' substance, on the other hand, did not vary appreciably during the experiment   The final extraction at 16 days was made on the remainder of the mass growth in order to obtain sufficient material for analysis of nitrogen distribution   It is interesting to note that the same average yields for the two substances were obtained from this large mass as when much smaller quantities were being manipulated   The method adopted to extract these large masses of vibrios will be detailed below

Experiments were performed on two strains to compare the amount of the acid-soluble substances obtained by using different concentrations of HCl in alcohol The results are given in Table IV —

<div align="center">

TABLE IV

Influence of concentration of HCl on the amount of acid-soluble substance extracted

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Strain	Concentration of HCl	Yield of ' A ', g	Concentration of HCl	Yield of ' B ', g
Rangoon, smooth	0 025 N	0 0012	0 05 N	0 0994
	0 125 N	No yield	0 25 N	0 1136
505	0 0125 N	0 0266	0 025 N	0 0522
	0 025 N	0 0288	0 05 N	0 0344
	0 125 N	No yield	0 025 N	0 0466

It is evident that ' A ' substance is susceptible to the higher concentrations of acid   The yield of ' B ' substance, on the other hand, does not appear to vary much with the concentrations of acid used in these experiments

In Table V we have given the percentage yields of the two acid-soluble substances from the twelve strains studied   The results obtained with the El Tor strain have already been given in Table III and are not repeated in Table V Altogether 30 extractions of ' A ' and 32 extractions of ' B ' were made —

<div align="center">

TABLE V

Percentage yields of acid-soluble ' A ' and ' B ' substances

</div>

Strain	Protein number	Character of growth on agar	' A ' substance, per cent	' B ' substance, per cent
1676	I	Smooth	3 2	10 1
Rangoon, smooth	I	Smooth	0 3	
			0 2	6 0
			0 2	6 2
			4 9	5 0
			5 0	6 3

TABLE V—*concld*

Strain	Protein number	Character of growth on agar	'A' substance, per cent	'B' substance, per cent
Rangoon, rough (2)	*	Rough	0 1	7 8
1617	1	Smooth	2 1	9 0
			2 8	6 0
			3 1	5 9
			4 2	6 4
				7 8
			3 3	4 0
			2 3	3 6
79A Euglobulin and pseudo globulin	I	Rough	3 1	5 4
79A	I	Rough	0 3	12 3
			1 0	4 0
2027	I	Smooth	0 4	6 1
E .	I	Smooth-rough	.	14 1
79B Euglobulin and pseudo-globulin	II	Rough	2 5	20 2
79B	II	Rough	0 44	6 6
W880 Euglobulin and pseudo globulin	II	Smooth-rough	3 8	21 8
W880 . .	II	Smooth rough	2 0	4 1
			1 1	7 0
			1 6	6 2
W3075 .	II	Smooth-rough	0 9	5 7
Basrah IV Euglobulin and pseudoglobulin	II	Smooth-rough	3 7	15 0
Basrah IV	II	Smooth-rough	0 7	3 2
El Tor Euglobulin and pseudo globulin	II	Smooth rough	6 1	9 2

* Protein number not yet determined

As Table V shows, the amount of ' B ' substance in a strain is always greater than the amount of ' A ' substance   The amount of ' A ' and ' B ' substances derived from the pseudo- and euglobulin fractions is usually considerably larger than that derived from the corresponding whole protein

Rough and smooth forms, as well as proteins of the two types, do not appear to differ significantly in the amounts of ' A ' and ' B ' present   Omitting from the account the amounts extracted from the purified pseudo- and euglobulins, ' B ' from Protein I averages 7 0 per cent while from Protein II it averages 5 0 per cent Protein I yields 2 1 per cent of ' A ' and Protein II 2 8 per cent, on the average With respect to type of growth the smooth forms have 6 3 per cent of ' B ', the smooth-rough forms 6 0 per cent and the rough forms 7 2 per cent, for ' A ' substance the averages are 2 4, 2 7 and 1 2 per cent respectively   It is possible that the development of a more exact technique for the extraction of ' A ' will show that the small amount in the rough strains is significant, and will at the same time allow us to determine whether any given strain shows a constant quantitative difference from other strains

Polarimetric readings —In this part of the work, the two acid-soluble fractions and the residue protein were dried at 37°C , weighed, and taken up in N/2 NaOH, according to the method already described (Linton, Mitra and Shrivastava, 1934a) The solutions were kept in the incubator at 37°C for two hours and the specific rotations then determined in a Schmidt and Haensch polarimeter, using sodium light   The results are shown in Table VI —

TABLE VI

Polarimetric readings on acid-soluble ' A ' and ' B ' fractions, residue protein and whole protein of cholera and cholera-like vibrios

Strain	Protein number	Character of growth on agar	Specific rotation in N/2 Alkali after 2 hours at 37°C			
			'A'	'B'	Residue	Whole
Rangoon, smooth	I	Smooth	—10 7	—47 7	—44 1	
				—48 2	—49 0	
					—44 6	
Rangoon, rough (2)	*	Rough		—48 8	—43 0	
1617	I	Smooth	—13 0	—52 0	—51 0	—56 0
				—44 3	—50 4	
79A Euglobulin	I	Rough	—11 3		—43 2	

* Protein number not yet determined

TABLE VI—*concld*

Strain	Protein number	Character of growth on agar	Specific rotation in N/2 Alkali after 2 hours at 37°C			
			'A'	'B'	Residue	Whole
79A	I	Rough	—10 9	—48 0	—48 5	
				—43 0	—52 0	
E Euglobulin	I	Smooth rough		—49 2	—50 0	
E Pseudoglobulin	I	Smooth-rough		—42 7	—54 0	
79B Euglobulin	II	Rough	—11 1	—46 0	—49 8	
79B Pseudoglobulin	II	Rough		—46 4	—50 1	
79B	II	Rough		—48 0	—52 0	
W880	II	Smooth rough	—14 8	—44 1	—47 0	—54 0
			—13 6	—44 3	—46 0	
				—48 4	—52 0	
W880 Euglobulin	II	Smooth rough		—49 1	—38 1*	
W880 Pseudoglobulin	II	Smooth rough		—44 1	—40 6*	
W3075	II	Smooth-rough		—44 7	—52 0	
Basrah IV	II	Smooth rough		—43 6	—49 0	
Basrah IV Euglobulin and pseudoglobulin mixed	II	Smooth rough	—10 3	—45 7	—43 0	
El Tor	II	Smooth-rough	—11 5	—42 8	—53 0	—53 0
			—13 0	—48 7	—45 0	
El Tor Euglobulin and pseudoglobulin mixed	II	Smooth rough	—12 3	—51 1	—46 0	

* Four-hour reading

Inspection of Table VI shows clearly that ' A ' substance is distinct in rotatory power from both ' B ' and the residue protein   Its mean rotation is about —12 0° ' A ' substance appears to have the same rotation whether derived from a smooth, smooth-rough or rough strain, or from Protein I or Protein II

In ' B ' substance, on the other hand, the rotatory power is much higher, varying from —42 7° to —52 0°, with a mean of —46 4°   These readings indicate a protein structure entirely different from that of ' A ' substance   ' B ' substance

strongly resembles the residue protein in rotatory power, the latter ranging from —43 0° to —54 0° with a mean value of —49 0° The two proteins thus show practically the same range of variation

That variations in rotation should occur in the extracts or in the residue protein is to be expected in view of the method used in preparing them As shown above, the fractions are not salt-free and were not made so since dialysis of such small amounts of material is impracticable It is clear from the table, however, that taking into consideration this disturbing factor, the range of variation in the respective fractions is not great, and that the differences between ' A ' on the one hand, and ' B ' and residue protein on the other, are marked enough to be significant

In a cholera vibrio strain (1617), a cholera-like vibrio strain (W880), and an El Tor strain, the specific rotations of the whole proteins were determined, and found to be —56 0°, —54 0° and —53 0° respectively These readings arc only slightly higher than those of the ' B ' substance and the residue protein Since No 1617 possesses Protein I and the other two strains Protein II, it is evident that the polarimetric readings of the whole proteins cannot be used to differentiate them

Rate of change in rotation —A further indication of the possible identity of ' B ' substance and residue protein is to be found in the rate at which the rotation changes in alkali Following Woodman (1921) we have already stated the evidence showing that identity in initial rotation and in rate of fall of rotation signify identity of structure (Linton, Mitra and Shrivastava, 1934a) The third identity, that of similar final rotation, could not be established in these experiments due to lack of material The average readings of the three fractions at 24-hour intervals are shown in Table VII The figures in parentheses indicate the number of determinations included in each average —

TABLE VII

Changes in specific rotatory power in protein fractions in N/2 alkali at 24-hour intervals

Time	' B ' substance	Residue protein	' A ' substance
2 hours	—46 5 (23)	—48 4 (23)	—12 0 (11)
24 ,,	—24 5 (13)	—25 0 (17)	—7 7 (2)
48 ,,	—18 9 (8)	—20 3 (14)	
72 ,	—16 2 (6)	—16 1 (7)	—4 0 (1)
96 ,,	—11 4 (1)	—11 4 (6)	

It is evident from the table that the two substances, ' B ' and residue protein, are very similar in the rate at which their rotatory power declines The readings for ' A ' substance are also included in the table and they indicate again the wide

difference between this fraction and the other two   It is interesting to note that all the readings in Table VII differ markedly from those given by purified Protein I and Protein II, thus indicating that the combination of ' A ', ' B ' and residue protein in the vibrios is quite different from any one of them separately

When the rate of change in rotatory power is considered on the basis of whether the fractions were derived from Protein I or Protein II we obtain the results given in Table VIII —

<div align="center">Table VIII</div>

Changes in specific rotatory power in protein fractions in relation to protein groups at 24-hour intervals

Time	Protein I ' B ' fraction	Protein II ' B ' fraction	Protein I Residue	Protein II Residue
2 hours	−47 1 (9)	−46 2 (14)	−44 5 (11)	−48 7 (12)
24 ,,	−25 1 (4)	−24 6 (9)	−25 0 (8)	−24 6 (9)
48 ,,	−19 9 (2)	−18 6 (6)	−19 3 (6)	−19 9 (8)
72 ,,	−16 5 (2)	−16 1 (4)	−16 0 (4)	−16 3 (3)
96 ,,	−11 4 (1)		−11 6 (3)	−11 2 (3)

The close resemblance between the four groups is obvious   Both in initial rotation and in rate of fall the ' B ' substances of Proteins I and II are very similar, and the residue proteins resemble each other and both the ' B ' substances throughout

Nitrogen distribution —In view of the small quantities of material (especially of ' A ' substance) available, we have determined the nitrogen distribution in these fractions by means of Hausmann's method (Plimmer, 1917), in which only 0 5 g to 1 0 g is required   To compare the results with those of the van Slyke method, the whole proteins were also done, and the results of the two methods were found to be completely in agreement   We have carried out the determination of nitrogen distribution on the fractions of three strains   No 1617, a typical cholera vibrio, which has Protein I, No W880, a non-agglutinating, cholera-like vibrio derived from water and having Protein II, and an El Tor strain, which also possesses Protein II   About 30 g (dry weight) of each vibrio was used   After removal of the flagellar material as described above, the vibrios were taken up in 1 litre of absolute alcohol made 0 025 N with HCl and extracted on the water-bath for 1½ hours at 50°C The organisms were removed from the alcohol, re-extracted in another litre, and the two supernatant fluids filtered and concentrated *in vacuo* to approximately 200 c c   The concentrate was precipitated with 3 volumes of ether, kept in the refrigerator overnight, removed and dried at 37°C

' B ' substance was then extracted in boiling 0 05 N HCl-75 per cent alcohol mixture   Three or four extractions of 15 minutes each were usually necessary in order to obtain sufficient material for the analyses   The first two were each made

in a litre and the last in 500 c c   After bringing the solution containing the ' B ' substance to the point of maximum precipitation with strong NaOH it was put in the refrigerator overnight, and the precipitate collected and dried at 37°C the next day   The percentage yields of ' A ' substance were found to be as high in these mass extractions as when small quantities of vibrios were being used   With ' B ' substance, on the other hand, it was more difficult to reach the percentages yielded with the smaller amounts

The results of Hausmann's analyses on the fractions is given in Table IX —

TABLE IX

Nitrogen distribution in acid-soluble ' A ' and   B ' fractions, residue protein and whole protein of cholera and cholera like vibrios

Strain	Fraction	Amide nitrogen, per cent	Humin nitrogen, per cent	Total bases nitrogen, per cent	Total phospho tungstic filtrate nitrogen, per cent	Total nitrogen, per cent	Nitrogen, per cent
1617	' A '	15 1	2 0	19 6	62 4	99 1	13 8
	' B '	7 5	4 7	23 6	67 2	103 0	13 4
	Residue	6 6	3 2	22 4	67 2	99 4	13 5
	Whole	7 2	4 4	24 7	64 9	101 2	13 6
W880	' A '	13 4	1 5	18 0	62 9	95 8	14 6
	' B '	7 4	4 1	23 2	68 4	103 1	13 3
	Residue	6 4	4 2	24 0	64 2	98 8	13 4
	Whole	6 7	4 4	24 1	64 8	100 0	14 2
El Tor	' A '	12 5	1 6	18 9	64 8	97 8	13 6
	' B '	7 3	4 7	23 8	65 1	100 9	13 4
	Residue	6 8	4 5	24 2	66 9	102 4	13 9
	Whole	6 8	4 4	24 7	62 5	98 1	14 0

The results given in Table IX are in concordance with those discussed above in showing that ' A ' substance is different from both ' B ' substance, residue protein, and whole protein   The amide nitrogen of ' A ' is twice as great as that of the other portions of the protein   Humin nitrogen is less than half that of the ' B ' substance, residue and whole protein   Total bases in ' A ' are also somewhat lower and are outside the range of variation previously found for the whole vibrio protein

The total phosphotungstic acid filtrate, which includes the mono-amino and non-amino nitrogen of the van Slyke method, is within the range of variation previously found

' B ' substance, residue protein and whole protein, on the other hand, do not differ in nitrogen distribution either from each other or from the whole proteins previously analysed by van Slyke's method

Toxicity —The ' A ' and ' B ' substances and the residue proteins were prepared in the usual way from cholera vibrio 1617 and cholera-like vibrio W880, except that the final precipitates were not dried, but were taken up in distilled water   Ten mg of each of the six fractions were injected intraperitoneally into guinea-pigs weighing about 250 g   During the first six hours after the injection, the animals were quiet and refused food   Within 18 hours the effect of the injected substances had com pletely disappeared and the animals became as active as the normal controls   No differences were apparent in the effect of the various fractions, and no symptoms of toxicity showed themselves in the animals

Ten mg of each of the six fractions were also injected subcutaneously into the skin of the shaved abdomen of an albino rabbit   In no case did any pronounced reaction occur until nearly 48 hours after the injection   The swelling and inflam-mation which were in evidence between 48 and 96 hours may safely be attributed to the foreign proteins as such and not to any special toxic quality, which would ordinarily tend to have its effect within a few hours   As in the case of the guinea-pigs, no differences appeared between the fractions from the cholera vibrio and the cholera-like vibrio   The ' A ' substances gave less reaction, which is in consonance with their simpler structure as shown by their lower rotatory power and their nitrogen distribution   But the reaction to ' A ' is of the same kind as to ' B ' and the residue protein (i e , a reaction to a foreign protein), though less in degree

DISCUSSION

In the present state of our knowledge of the vibrio proteins we are probably not justified in concluding that the ' A ' and ' B ' fractions are distinct chemical entities which the vibrios possess   As Gortner (1929) states, ' the mere fact that one can repeatedly isolate a given quantity of protein from biological material, using a rigidly standardized technique, is no valid proof that the material which is isolated constitutes a chemical entity '   We should rather hold the view that the ' A ' and ' B ' fractions are simply those portions of the whole vibrio protein which are soluble under the given conditions   Gortner (*loc cit*) and his co-workers have shown that relatively slight modifications in the technique of extracting protein fractions from wheat-flour will bring about marked differences in these fractions, and there is no reason to suppose that the same would not be true for vibrio proteins This point of view appears to hold in the present experiments especially for ' B ' fraction, which in specific rotatory power, rate of change in rotatory power and in nitrogen distribution, is very close to the residue protein   ' B ' substance would accordingly be that aliquot portion of the whole protein which is soluble under the conditions which we have used   If some part of the technique is changed, as when the more soluble globulins are used as the source of ' B ', this substance appears in increased amount   ' A ' substance, which differs markedly from ' B ' and residue protein in all the experiments, may be found to be a separate chemical entity   The

serological study of these fractions which is now in progress should enable us to answer this question

Our chief interest, however, in this paper as in the others in this series, is to compare the constituents of the cholera vibrios and the cholera-like vibrios   The experimental evidence which we have given above points directly to the chemical identity of the similarly designated fractions from the two types of protein   No chemical differences could be found between the ' A ' substances derived from Protein I and Protein II , they were indistinguishable in percentage yield, in rotatory power and in nitrogen distribution   The ' B ' substances from whatever protein source appeared also to be identical, and at the same time they are closely allied to the residue proteins   Furthermore, we have already shown that the nitrogen distribution in the whole proteins of the two groups is the same   While this evidence cannot be considered conclusive until the serological work is completed, it all points to the same tentative conclusion, namely, that Protein I and Protein II differ because of the arrangement of their constituents, and that these constituents are the same in both groups

SUMMARY

Two acid-soluble protein substances, designated ' A ' and ' B ', have been isolated from a group of vibrios in which the following strains were included cholera vibrios, cholera-like vibrios, smooth, smooth-rough, and rough vibrios   The proteins of these vibrios belonged about equally to Protein I and Protein II as defined in our earlier work (Linton, Mitra and Shrivastava, 1934a)

' A ' substance, which dissolves out of the vibrios at 50°C in alcohol made 0 025 N with HCl, has a specific rotation of about —12 0° (in N/2 alkali after 2 hours at 37°C) and a nitrogen distribution which is characterized by the possession of about twice the amount of amide nitrogen and half the amount of humin nitrogen occurring in the whole protein   ' A ' occurs in both the pseudo- and euglobulin fractions of the protein, appears to increase when the whole protein is kept for two weeks in distilled water at 10°C , and is destroyed when extraction is attempted in 0 125 N HCl   Its yield is usually about 1 to 2 per cent of the dried weight of the vibrios, although we were unable to arrive at completely constant results in respect to the amount extracted

' B ' substance was obtained after the removal of ' A ' by boiling the vibrios for 15 minutes in 75 per cent alcohol, diluted with normal saline, and made 0 05 N with HCl   The material removed by this method has a rotatory power of about —46 0° and a nitrogen distribution indistinguishable from that of the whole vibrio protein   ' B ' is found in both the globulin fractions, and, like ' A ' substance, comes in larger amounts from the euglobulin   Its yield does not appear to be affected by high concentrations of HCl, nor does it increase in vibrio protein kept in the refrigerator in distilled water   In general, ' B ' substance is about 6 per cent of the dry weight of the protein, but as in ' A ' the percentage yields are not constant

A comparison of all the chemical findings indicates that the ' A ' substance is very similar from whatever type of strain or protein it is extracted   ' B ' substance differs markedly from ' A ', but again is the same irrespective of source   ' B ' is closely allied in composition to the residue protein which remains after the two

acid-soluble fractions have been removed, and is also identical with the whole protein, in so far as chemical methods allow us to judge

None of the fractions is toxic when given intraperitoneally to guinea-pigs, nor do they give rise to skin reactions indicative of toxicity when administered subcutaneously to rabbits

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NOTES ON THE STRUCTURE OF THE CHOLERA AND CHOLERA-LIKE VIBRIOS

BY

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THE ELEMENTARY CONSTITUENTS OF THE CHOLERA AND THE CHOLERA-LIKE VIBRIOS

THIS note deals briefly with a quantitative analysis of the elementary constituents of the vibrios, the percentages of fat in the vibrios, and the analysis of the ash of vibrio proteins   The four groups into which the vibrios are divided in the table represent the division of these organisms on the basis of their protein and carbohydrate structure which we have put forward elsewhere (Linton, Mitra and Shrivastava, 1934*a*)   The method for the purification of the proteins and for the extraction and determination of the fat content has already been described (Linton, Mitra and Shrivastava, 1934)

TABLE

Percentages of fats and of some elementary constituents in the cholera and cholera-like vibrios

Vibrio group number		Fats	Carbon	Hydrogen	Sulphur	Phosphorus	Nitrogen	Ash
I	Rangoon S				0 66	0 56	14 97	2 42
	Rangoon R		51 46	8 33	0 64	0 59	14 77	2 42
	1617		51 57	8 32	0 67	0 53	14 45	2 30
	454				0 67	0 60	14 46	
	1612	2 05						

TABLE—*concld*

Vibrio group number		Fats	Carbon	Hydrogen	Sulphur	Phosphorus	Nitrogen	Ash
II	505		50 10	7 93	0 62	0 60	14 68	3 04
	E	2 30				0 55	14 35	
III	Basr th IV	2 29	51 06	8 66	0 58	0 56	13 95	2 32
	W3075	2 20			0 60	0 57	14 64	2 29
	W880				0 61	0 53	13 75	2 40
	25	2 79				0 49		
	W467	2 74				0 50		
	W785	2 68				0 60		
IV	El Tor	2 80	51 76	8 56	0 64	0 61	13 83	2 67
	79B	2 50			0 66	0 56	14 35	

Protein weighing 44 05 g , derived from the above vibrios, was ignited The yield of ash was 1 21 g (2 75 per cent) and its composition was as follows —

	Per cent		Per cent
PO₄	35 81	K	6 75
Fe	0 08	Na	33 10
SO₄	2 16	Cl	19 98
Mg	Trace	Ca	1 03

Previous study on the aspect of the vibrios treated in this note appears to be limited to the work of Cramer (1895, 1897) and of Nicolle and Alilaire (1909) The latter workers included one vibrio among the organisms which they studied Cramer made the important discovery that the elementary composition of the vibrios varied with the medium upon which they grew He showed that the vibrios did not have a characteristic elementary composition and that the inorganic content could be changed at will by varying the composition of the substrate

All the five strains which Cramer used were of human origin It was obviously impossible at that time to have studied them in relation to bacterial variation

Our purpose here has been to study a larger series than his, drawn from a variety of sources, and with known carbohydrate and protein composition   A comparative study of the cholera and the cholera-like vibrios, such as we are making, would not be complete without a consideration of the elementary composition of the members of the two groups

It is evident from the table that the vibrios do not differ significantly among themselves in any of the constituents which we have studied   All contain about 0 5 per cent of phosphorus and a slightly greater percentage of sulphur   Carbon, hydrogen and nitrogen show the values usually obtained for normal proteins, and the fat content of about 2 5 per cent is that found for most bacteria, except those belonging to the acid-fast group   The average figure for nitrogen is 14 0 per cent which is equivalent to a protein content of 87 5 per cent   As we have already shown, the specific carbohydrate content is variable, depending upon the type of growth, but is in general 6 to 8 per cent of the vibrio body   The figure for ash of 2 5 per cent on the average is calculated at the minimum and would be somewhat larger if the state in which the constituents exist in the protein were taken into account   These figures, together with the average figure of 2 5 per cent for the fat content, make up the total very close to 100 per cent, and allow us to conclude that any other possible constituents of the vibrio body must be present only in small amounts

An analysis of the polysaccharide of a group III vibrio

Vibrio W880, which we received through the courtesy of Captain C L Pasricha, i m s , was isolated from the Hooghly River in June 1933   It is morphologically a typical vibrio, not agglutinable with anti-cholera serum, produces indol and is smooth to Millon's test   It is reported to be lysable by a vibrio 'phage derived from water

Our previous work had shown that it was composed of protein II, and that the characteristic sugar in its polysaccharide was arabinose   It, therefore, fell into our vibrio group III, which contains all the water-vibrios we have analysed up to the present   Our purpose in the present experiments was to study its specific polysaccharide more closely, according to the methods we have used previously (Linton and Shrivastava, 1933, 1933a)

Three and one-half g of the purified specific polysaccharide were added to 5 c c of H_2SO_4 diluted 1   1 and the mixture placed at 37°C   for 20 hours This mild treatment resulted in the appearance of 18 per cent of reducing substances, calculated as glucose   Since it was believed that arabinose, which is easily destroyed by acids, was present we did not hydrolyse further by stronger methods

The sulphuric acid was removed from the hydrolysate by $CaCO_3$, and the calcium salt of the aldobionic acid prepared and precipitated out by the addition of alcohol   The supernatant fluid was concentrated and a phenylosazone prepared from it   The purified crystals had a melting point of 158°C to 160°C   The known arabinose compound melts at 160°C

best fixative for demonstration of acid-fast bacilli, but it causes much shrinkage of the tissue

(b) *The treatment of the section before staining* —The removal of paraffin by xylol and of xylol by alcohol has an effect on the subsequent staining of bacilli Either xylol or alcohol acting separately has a slightly harmful effect, but xylol followed by alcohol has a still greater effect   We have found it advisable to use xylol only, omitting the use of alcohol, and removing the excess of xylol by thorough blotting and rapid drying by waving the slide in the air

(c) *Staining with carbol fuchsin* —While in staining smears, heating for 10 minutes or less in 1 per cent carbol fuchsin is satisfactory, in sections we have obtained far better results by using stronger (4 per cent) carbol fuchsin at room temperature for longer periods (30 minutes)   Apparently the reduced acid-alcohol-fastness makes stronger staining necessary

(d) *Decolorization of tissues after carbol fuchsin* —Since the bacilli in sections have very little alcohol-fastness, watery acids, and not acid-alcohol, must be used for decolorization

(e) *Counterstaining* —For similar reasons counterstains must not contain much alcohol, preferably none at all, and they should be used for as short a time as possible

(f) *Dehydration after staining* —Alcohol must not be used   We have found blotting with filter-paper and drying in air satisfactory

A SATISFACTORY METHOD

The tissue is fixed in 70 per cent alcohol, 10 per cent formalin or Zenker's fluid It is dehydrated as quickly as possible , 70 per cent alcohol for 24 hours, 95 per cent alcohol for 6 hours absolute alcohol 12 hours, is usually sufficient   The tissue is then cleared and embedded as usual, and sections are cut   The sections are floated on to slides which have previously been smeared with a trace of egg-albumen fixative (white of egg 50 c c , glycerin 50 c c , sodium salicylate 1 gramme) and the slides are placed overnight in an incubator   The paraffin is removed by xylol (3 changes) and the section is then blotted with clean filter-paper, and dried in the air for a few seconds , it is then washed in water   (In sections fixed in Zenker's fluid the mercury must then be removed by Lugol's solution)

A small piece of filter-paper about three-quarters of an inch square is then placed over the section, and Kinyoun's carbol fuchsin* is filtered on to the slide The filter-paper prevents deposition of stain on the section and ensures that the section remains covered by the stain   The stain is left on at room temperature for half an hour, and then washed off with water   The slide is then dipped into 5 per cent acid (HCl or H_2SO_4) in water for a period varying usually from 5 to 20 seconds according to the thickness of the section   As soon as most, but not all, of the red has disappeared, the slide is removed and washed in water†   It is then

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* Basic fuchsin 4 g , phenol crystals 8 g , alcohol 95 per cent 20 c c , water 100 c c
† At this point the slide may be dipped for one second only into acetone and washed again in water   This procedure completes the decolorization of the tissue without decolorizing appreciably the bacilli and thus cleans up the section very well   This procedure is not always necessary, but it is often very useful

stained for about half a minute only in Delafield's hæmatoxylin and again washed in water   To improve the nuclear staining the section is then dipped for one second only in 1 per cent acid in water, washed, and then dipped for a few seconds in weak ammonia water and washed again thoroughly   As a cytoplasmic counterstain a solution of orange G in water* is used for a few seconds   Without washing the section is blotted with filter-paper, dried rapidly in air, cleared in xylol and mounted in neutral balsam

This method used with care and judgment is capable of giving beautiful results, and with a little experience it is found that these results are very constant   Failures are practically eliminated   Tissues which when stained by the common methods show no or few bacilli, when stained by this method often show some or many bacilli   The percentage of positive findings in tissues examined has greatly increased, and the number of bacilli found in positive lesions has also greatly increased

It is true that for cell-staining this method is not ideal, but on the whole it is surprising what good staining of nuclei and cells is obtained   A study of the nature of the cells and of the relation of the bacilli to the cells is usually quite easy   If more critical cell-staining is desired it is usually necessary to cut serial sections, and to stain one section for bacilli and the next for cells

If the section is being examined merely for the presence of *M lepræ*, good cell-staining not being required, the method described may be modified as follows   The section after decolorization is counterstained for a few seconds with weak methylene blue, washed in water, blotted, dried, cleared in xylol, and mounted in balsam

(*Note* —Since making these investigations, we have seen an unpublished paper by Dr H W Wade, previously Pathologist of the Leper Colony, Culion, Philippine Islands, in which he mentions some of the points discussed here, and advocates a somewhat similar procedure for staining *M lepræ* in sections)

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* Four c c of saturated watery orange G to 100 c c of water

BACTERIOPHAGE, ESSENTIAL OILS AND VACCINATION AND THEIR EFFECTS ON CHOLERA MORTALITY.

BY

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CHOLERA broke out in the Silchar and Hailakandi sub-divisions of Cachar in September 1933   By the second week of October it was widely distributed in both areas and the opportunity was taken to try and stem the epidemic by vaccination and by treating the cases with essential oils in Silchar, and with bacteriophage alone in Hailakandi

In Silchar, vaccination of contacts and treatment of cases with essential oils had been carried out from the beginning of the epidemic   Five thousand inoculations had been done by the 8th October, 1933, by which time there had been 349 cases, i e , 14 were vaccinated for each case , but vaccination on a large scale began on the 11th October, 1933, and was carried out by R A H and a staff of forty assistant and sub-assistant surgeons   In four weeks 98,827 inoculations were given

Hailakandi was entrusted to one of us (E M R) on the 17th October, 1933, and he with five assistants had distributed bacteriophage to the 316 villages in the sub-division by the 22nd October, 1933

Bacteriophage had not been used in Cachar before and there was some hostility shown to the experiment   As a *placebo*, vaccination was permitted

in Hailakandi after the 26th October when it was expected that the results of the 'phage experiment would be evident   The 'phage staff did not vaccinate but the official returns claim that 15,608 vaccinations were done by the district officers in Hailakandi between the 8th October, 1933, and the 25th November, 1933

The areas under consideration are two parallel and adjacent valleys running north and south   They contain streams which flow north to enter the Barak river The population of Silchar is 386,695 and that of Hailakandi is 150,992   In both valleys the cholera was confined to villages along the river banks so that the populations at risk were not so divergent as the census figures would indicate   One of us (R A H) puts the populations of the infected villages at 110,000 and 67,000 respectively

The people of these valleys are similar in caste, in housing and in habits   There was no selection of cases for treatment in either valley   Whether a case was vaccinated and got essential oils or was given bacteriophage depended on which remedy was available in the village   Records were obtained on the form in the Appendix   It was not possible to scrutinize these records on the spot to the extent done in our previous work in Sibsagar and Darrang but, frequently, villages were visited twice or oftener by different medical officers and duplicate or triplicate reports were received   These are remarkably consistent, varying only in unimportant details   Where the dates differ, those given by the medical officer who visited the house within a day or two of the cases have been taken   Cholera vibrios were obtained from cases in both valleys and sera from convalescent cases   The results of agglutination tests with the sera are given in Tables I, I-A and I-B —

TABLE I

Area	Negative	1/25	1/50	1/100	1/200	1/300	1/500	1/750	1/1,000	TOTALS
Silchar	38	2	19	5	9	2	4	8	4	91
Hailakandi	33	4	14	15	15	5	0	7	2	95
TOTALS	71	6	33	20	21	7	4	15	6	186

Table I-A

Serum agglutinations, Silchar sub-division

Days	1,000	750	500	300	200	100	50	25	0
1							1		1
2							1		2
3		1			1				4
4	1				1		1		4
5					2	1	1		2
6	1			1				1	2
7	1	1		1					1
8	1	1		1	1				1
9				1		1			1
11			2	1		1			1
13									1
15		1							
18									1
19					1				
32									1
40					1				1
41						1	1		1
43						1			1
46		1							1
48		1	1						1
49									1
50						1			2
51						1	1		1
53			2						1
54									1
56									2
57		1		1					
58						1			
61				1		1			1
62				1					1
63					1				
64						1			
66									2
67						1			
68						5			
70					1				1
72		1				1			1
84						1			

Number of days since attack

TABLE I-B

Serum agglutinations, Hailakandi sub-division

Dilutions of sera	4	5	8	10	12	14	20	22	24	25	26	28	29	30	31	32	34	35	36	37	38	39	40	41	42	43	44	45	46	47	49
1,000				1								1																			
750					1			1							1					3					1						
500																															
300															1	1	1										1				1
200						1									1					2		2	2	2		1					1
100		1					1	1			1	1				1		1		2	2	1		1	2	1		1			
50												1				1			4	1	2	1	1								
25															1		1			1	1						2				
0	1		1						1	2	2		2	1	1	1	1	1	6	2	2	1	2			2			1	1	1
Days	4	5	8	10	12	14	20	22	24	25	26	28	29	30	31	32	34	35	36	37	38	39	40	41	42	43	44	45	46	47	49

Number of days since attack.

The 17th October, 1933, was the first day on which bacteriophage was given to any extent (Graph 1)   The mortality in each area before and after that date is shown in Table II and Graphs 2 and 3 —

GRAPH 1

Graph 2.

CHOLERA 1933
SILCHAR
SUB DIVISION
RECOVERIES ☐   DEATHS ■

Graph 3.

MORTALITY
CHOLERA 1933
HAILAKANDI
SUB DIVISION
RECOVERIES ☐   DEATHS ■
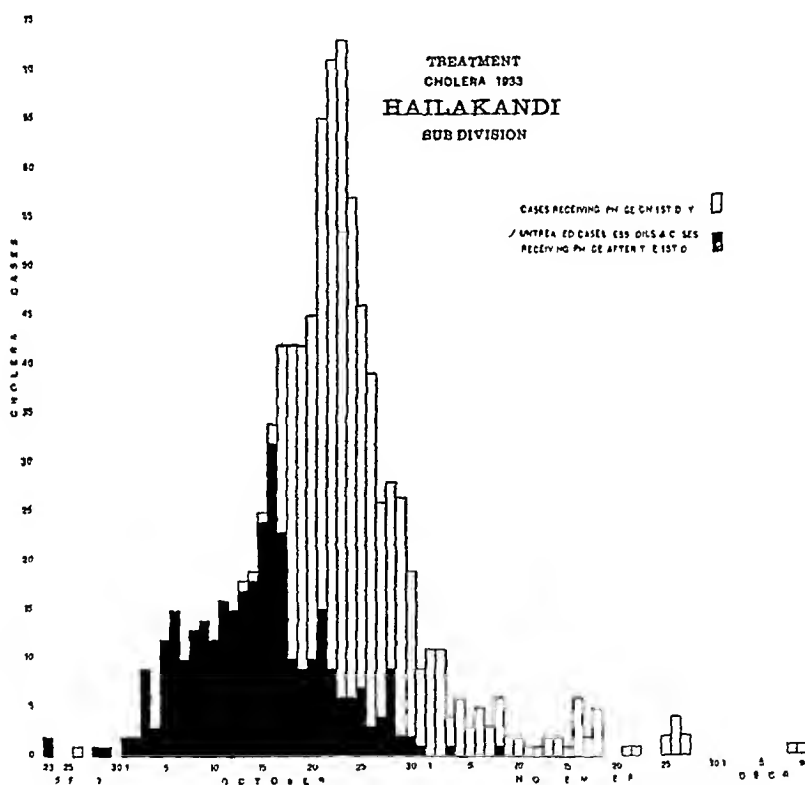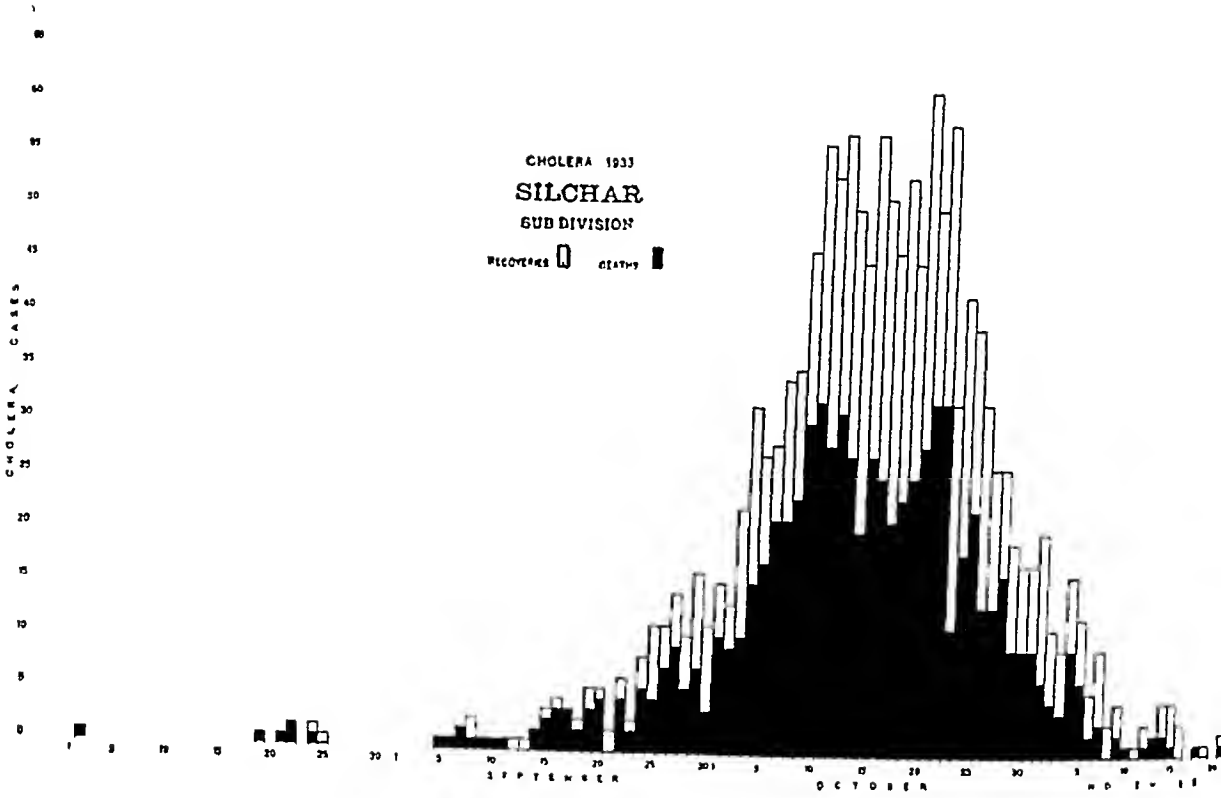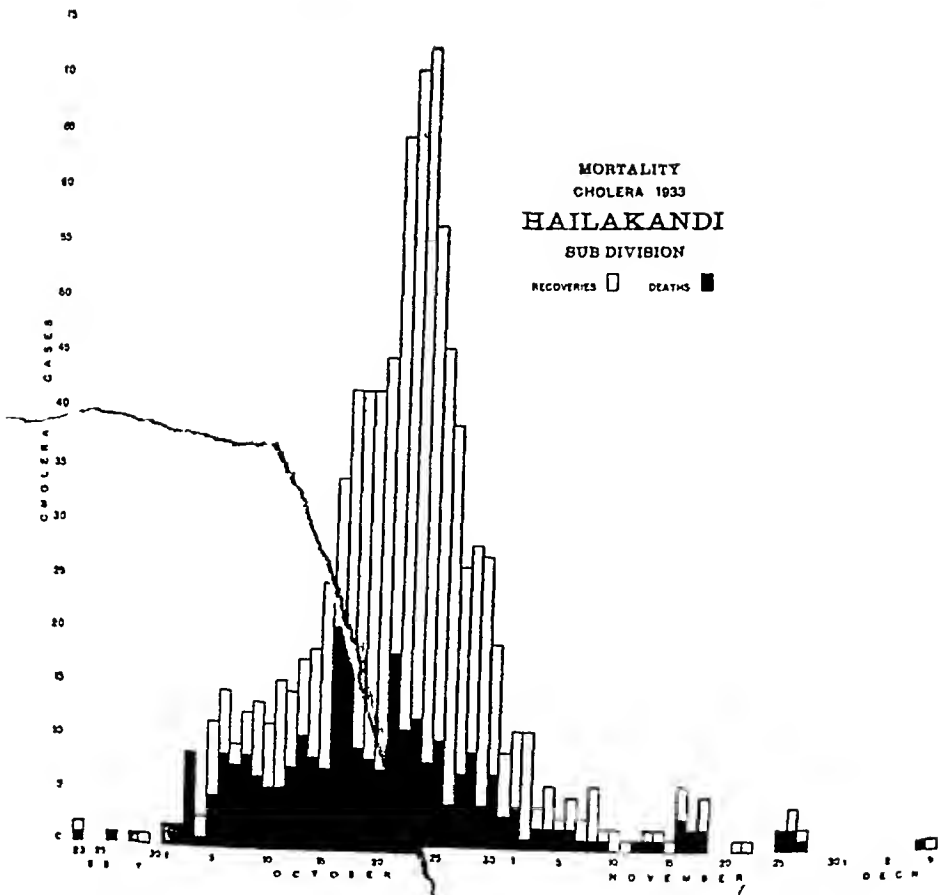
TABLE II

Area	BEFORE 17TH OCTOBER, 1933			ON AND AFTER 17TH OCTOBER, 1933		
	Cases	Deaths	Mortality	Cases	Deaths	Mortality
Silchar	787	454	57 7	701	334	49 1
Hailakandi	224	124	55 4	711	170	23 9

The epidemic began in Silchar about a fortnight before it began in Hailakandi , allowing for this the first parts of the ascent of the graph for each area are alike 'Phage was begun in Hailakandi when the ascent was most rapid, i e , between the 17th and the 22nd October, 1933

Both epidemics ended in the fourth week of November 1933, except for two cases which occurred in Hailakandi in December   In Table III the treatments which were given in each valley are analysed —

TABLE III

Treatment of cholera in Cachar, 1933

Treatment	SILCHAR			HAILAKANDI			Total cases	Mortality
	Cases	Died	Percentage	Cases	Died	Percentage		
No treatment	804	551	68 5	154	120	77 9	958	70 04
Vaccinated only	67	39	58 2	2	1		69	57 9
Vaccinated a n d essential oils	83	21	25 3	9	2		92	25 0
Vaccinated no date and essential oils	2	1					2	
Vaccinated and bacteriophage	8	0		56	12	21 4	64	18 7

TABLE III—*concld*

Treatment	SILCHAR			HAILAKANDI			Total cases	Morta lity
	Cases	Died	Percentage	Cases	Died	Percentage		
Vaccinated, essen t i a l o i l s and bacteriophage			.	1	1		1	
Essential oils only	481	176	36 6	51	24	47 1	532	37 6
Essential oils and saline	1	0		0	0		1	
Essential oils and calomel	1	1		0	0		1	
Essential oils and bacteriophage	1	1		10	2		11	
Saline and perman ganate of potass	2	0		1	0		3	
B a c t e r i o phage within 48 hours	24	5	20 8	617	131	21 2	641	21 2
B a c t e r i o phage after 48 hours	9	1		29	3		38	10 5
B a c t e r i o p hage within 48 hours and saline				3	1		3	
Chlorodyne	4	2		0	0		4	
Calomel	1	0		2	1		3	
TOTALS	1,488	798	53 6	935	298	31 9	2,423	45 23

Before studying these mortalities we may examine how far the intended differentiation of the two valleys by treatment was attained   The population of Silchar is 386,695 and that of Hailakandi is 150,992 but one of us (R A H) estimated the

population of the infected villages in Silchar to be 110,000 and in Hailakandi to be 67,000   Before accepting this we applied Colonel Russell's estimate that in Madras in the same houses the incidence of cholera was 0 41 and 2 1 per cent among inoculated and non-inoculated respectively   Using these percentages and the figures in Table III the population at risk in Silchar and Hailakandi were 102,260 and 57,900 respectively   The figures given by R A H may, therefore, be accepted as a reasonable approximation if the incidence of cholera in both inoculated and non-inoculated was slightly lower than in Madras

TABLE IV

Distribution of treatments	SILCHAR			HAILAKANDI			TOTALS
	Observed	Expected	χ^2	Observed	Expected	χ^2	
Escaped cholera	108,512	108,494 176	0 003	66,065	66,082 817	0 0049	174,577
No treatment	904	595 367	73 111	154	362 633	120 032	958
Vaccination	160	141 695	2 365	68	86 305	3 882	228
Essential oils	484	338 701	62 332	61	206 299	198 607	545
'Phage	33	423 842	360 411	649	258 158	591 721	682
Sundry	7	6 215	0 099	3	3 785	0 163	10
TOTALS	110,000			67,000			177,000

Table IV shows the distribution of treatments compared with what might be expected in a distribution proportionate to the population   In both valleys the number attacked and the number who escaped approximate to what might be expected in a proportional distribution   The untreated were 209 or 35 per cent in excess and the same number or 58 per cent in defect in Silchar and Hailakandi respectively   There was less difference between the two valleys in the inoculated   Of those at risk Silchar had only 18 cases or 13 per cent more than might be expected and Hailakandi had 18 or 21 per cent less   The actual figures for vaccination from 7th October to 25th November, 1933, are 105,983 and 15,608 respectively   If all the Hailakandi vaccinations were persons at risk then about 39,000 of the 105,983 vaccinations in Silchar may also have been people at risk

In the essential oils group the difference between observed and expected is 145   These cases were in excess by 43 per cent in Silchar and in defect by 70 per cent in Hailakandi

Bacteriophage cases were 92 per cent in defect in Silchar and 150 per cent in excess in Hailakandi   In spite, therefore, of the extensive vaccinations done in Silchar the real difference, if this way of looking at it is correct, lay in the bacterio phage, to a less but significant extent in the use of essential oils or of no treatment and comparatively little in vaccination   We do not seem to have a vaccinated and unvaccinated area though the figures for vaccination in Table V give this impression —

<div align="center">

TABLE V

Village vaccinations, Cachar district

</div>

Week ending	Silchar	Hailakandi
October 14th, 1933	12,446	243
„ 21st, 1933	28 606	1,236
„ 28th, 1933	36,349	2,805
November 4th, 1933	21,426	6,828
„ 11th, 1933	6,346	3,912
„ 18th, 1933	810	166
„ 25th, 1933		418
TOTALS	105,983	15,608

In Table VI, 804 untreated cases in Silchar show a mortality of 68 5 per cent and 154 cases in Hailakandi, a mortality of 77 9 per cent   The mortality for males in Silchar was 65 7 per cent and in Hailakandi 77 2 per cent   For females the Silchar mortality was 71 5 per cent and in Hailakandi 78 7 per cent   The age-group mortalities are remarkably consistent   The standard deviations of the mean mortalities are 4 9 and 2 09 respectively   The relatively low mortality of 43 9 for male children under 5 years of age in Silchar is the chief factor in reducing the Silchar mortality   In Silchar 68 3 per cent of the deaths occurred within 48 hours, in Hailakandi 71 per cent

When the untreated cases are distributed in weekly periods Tables VII and VIII show that the mortality in Silchar varied from 54 7 to 85 7 per cent, in Hailakandi from 73 6 to 82 7 per cent (grouping the last month)   Whether the mortality in Hailakandi is compared with the corresponding week in Silchar or with the figures a fortnight later is immaterial, the tendency is for the Silchar figure to be less than the Hailakandi   The difference for cholera is so little that we are not justified in taking the disease to be of different virulence in the two areas   In neither Tables VII nor VIII is there any evidence of a lower mortality towards the end of the epidemic

TABLE VI

Mortality by age and sex
Untreated cases

Age groups	SILCHAR SUB DIVISION								HAILAKANDI SUB DIVISION								
	MALES			FEMALES			TOTALS		MALES			FEMALES			TOTALS		
	R	D	Mortality	R	D	Mortality	Cases	Mortality	R	D	Mortality	R	D	Mortality	Cases	Mortality
0–4	32	25	43 9	16	24	60 0	97	50 5	1	9	90	2	10	83	22	86 3
5–9	36	65	64 4	22	59	72 8	182	68 1	4	12	75	4	8	66	28	71 4
10–19	20	50	71 4	15	59	79 7	144	75 7	3	13	81	5	7	58	28	71 4
20–29	25	42	62 7	41	73	64 0	181	63 5	6	12	66	2	7	78	27	70 3
30–39	19	33	63 4	13	33	71 6	98	67 3	2	10	83	1	9	90	22	86 3
40–49	3	21	87 5	3	14	82 3	41	85 3	2	4	66	0	7	} 100	13	84 6
50–59	1	17	} 86 5	0	11	} 87 5	29	} 86 8	2	5	} 90	0	2		9	} 85 7
60–69	4	11		2	8		25		0	3			2		5	
70 and over	0	4		1	2		7									
TOTALS	140	268	65 68	113	283	71 46	804	68 53	20	68	77 2	14	52	78 7	154	77 9

R.—Recovered   D.—Died

Standard deviation 4 9

Standard deviation 2 09

Days on which deaths occurred

Silchar sub division

Days	1	2	3	4	5	6	7	8	9	10	12	19	TOTALS
Cases	218	158	81	34	25	12	13	5	1	2	1	1	551
Percentage	39 6	28 7	14 7	6 2	4 5	2 2	2 4	0 9	0 2	0 4	0 2	0 2	100 2

Hailakandi sub division

Days	1	2	3	4	5	6	7	9	10	11	12	TOTALS
Cases	56	31	11	7	4	1	3	1	4	1	1	120
Percentage	46 7	25 0	10 0	5 8	3 3	0 8	2 5	0 8	3 3	0 8	0 8	99 8

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Bacteriophage cases were 92 per cent in defect in Silchar and 150 per cent in excess in Hailakandi   In spite, therefore, of the extensive vaccinations done in Silchar the real difference, if this way of looking at it is correct, lay in the bacterio phage, to a less but significant extent in the use of essential oils or of no treatment and comparatively little in vaccination   We do not seem to have a vaccinated and unvaccinated area though the figures for vaccination in Table V give this impression —

TABLE V

Village vaccinations Cachar district

Week ending	Silchar	Hailakandi
October 14th, 1933	12 446	243
„ 21st, 1933	28,606	1,236
„ 28th, 1933	36,349	2,805
November 4th, 1933	21,426	6,828
„ 11th, 1933	6,346	3,912
„ 18th, 1933	810	166
„ 25th, 1933		418
TOTALS	105,983	15,608

TABLE VIII

Mortality by age and period, Hailakandi sub-division

Untreated cases

Age group	Sept		Oct 1-7		Oct 8-14		Oct 15-21		Oct 22-28		Oct and Nov 20-4		Nov 5-11		Total cases	Mortality
	R	D	R	D	R	D	R	D	R	D	R	D	R	D		
0-4	0	1	1	4	0	3	1	6	1	2			0	1	22	86 3
5-9	1	0	1	6	3	5	4	4	0	1					25	74
10-19			2	4	4	8	1	7	0	1					28	71 4
20-29	0	1	3	5	5	7	0	7							27	70 3
30-39			1	5	0	7	2	6			0	1			22	86 4
40-49	0	1	1	4	0	4	1	2							13	84 6
50-59			0	2	2	1	0	4							9	85 7
60 and over			0	1	0	1	0	3							5	
TOTALS	1	2	9	31	14	39	9	39	1	7	0	1	0	1	154	77 92
Mortality			76 4		73 6		81 25				90 0					

R —Recovered   D —Died

TABLE IX

Mortality by age and sex

	SILCHAR SUB DIVISION ESSENTIAL OILS								HAILAKANDI SUB DIVISION 'PHAGE WITHIN 48 HOURS							
	MALES			FEMALES			TOTALS		MALES			FEMALES			TOTALS	
Age groups	R.	D	Mortality	R.	D	Mortality	Cases	Mortality	R.	D	Mortality	R.	D	Mortality	Cases	Mortality
0—4	13	6	31 5	9	12	57 1	40	45 0	36	6	14 2	28	10	26 3	80	20 0
5—9	29	22	43 1	41	11	21 1	103	32 0	62	12	16 2	39	18	31 5	131	22 0
10—19	22	9	29 0	32	17	34 7	80	32 5	49	13	25 0	46	14	23 3	122	22 1
20—29	38	13	25 4	52	28	35 0	131	31 3	48	12	20 0	66	15	18 5	141	19 1
30—39	25	14	35 9	13	14	51 8	66	42 4	31	7	18 4	27	6	18 1	71	18 3
40—49	11	8	42 1	9	4	30 6	32		16	4	20 0	10	3	23 1	33	21 2
50—59	2	10	} 63 7	3	6	} 53 8	21	} 49 2	10	4	} 31 8	9	2	} 23 5	25	} 23 2
60—69	3	1		3	1		8		4	3		3	2		12	
70 and over	0	0		0	0		0		1	0		1	0		2	
TOTALS	143	83	36 7	162	93	36 4	481	36 6	257	61	19 2	229	70	23 4	617	21 2
	Standard deviation 4 02								Standard deviation 3 33							

R.—Recovered. D—Died

Days on which deaths occurred

ESSENTIAL OILS

Days	1	2	3	4	5	6	7	8	9	10	12	15	16	22	TOTALS
Cases	14	37	40	10	13	6	2	5	2	1	2	3	1	1	176
Percentage	25 0	21 0	22 7	10 8	7 4	3 4	1 1	2 8	1 1	0 6	1 1	1 7	0 6	0 6	99 2

'PHAGE WITHIN 48 HOURS

Days	1	2	3	4	5	6	7	8	9	10	11	12	13	15	TOTALS
Cases	36	34	24	11	5	5	3	4	2	2	1	1	1	2	131
Percentage	27 5	25 95	18 3	8 4	3 8	3 8	2 29	3 05	1 5	1 5	0 76	0 76	0 76	1 5	99 84

In Table IX the essential oils cases in Silchar and the 'phage cases in Hailakandi are arranged by age and sex   Among 481 cases given essential oils the mortality is 36 6 per cent and for 617 cases given 'phage it is 21 2 per cent   The standard deviations are 4 02 and 3 33 respectively

Untreated groups are weighted by cases that die within a few hours before they can get treatment and treated groups have correspondingly fewer deaths   Were it possible to take alternate cases the moment they fell ill this would not happen, but, in a rapidly fatal disease like cholera, where out of 958 untreated cases 274, or 28 6 per cent, die on the day they fall ill, this must happen   How far this has happened in Silchar we cannot estimate for the untreated were 804 and the cases having essential oils 481   But in Hailakandi it does not matter   Before the 17th October in Hailakandi there were 45 cases receiving only essential oils with 22 deaths   Among the 'phage cases there were 8 treated on the first day, 16 on the second day and 12 later   All the last 12 recovered , they may have been on the way to recovery before receiving 'phage and we may transfer these 12 to the untreated group   With this addition the untreated in this period were 133 with 92 deaths giving a mortality of 69 per cent for the untreated, 48 9 per cent for the essential oils and 16 7 per cent for the 'phage groups respectively

Table X

Analysis of cases in Hailakandi before 17th October

Treatment	R	D	Treatment	R	D
Essential oils	23	22	Vaccinated	1	0
Essential oils and 'phage within 48 hours	3	1	Vaccinated and essential oils	7	1
Essential oils and 'phage after 48 hours	3	1	Vaccinated and 'phage after 48 hours	0	2
			Chlorodyne	1	1
'Phage on 1st day	7	1	Untreated	29	92
„ 2nd „	13	3			
„ later	12	0			

R —Recovered   D —Died

After the 16th October in Hailakandi there were only 33 untreated cases   This illustrates the rapidity with which 'phage can be distributed to a large area   If we transfer these 33 untreated cases with their 28 deaths to the 'phage group and deduct the above 12 recoveries the weighted mortality for 638 cases is 24 9 per cent as compared with 36 6 per cent in the essential oils group in Silchar, the latter being unweighted

Essential oils treatment was not available in Silchar as 'phage was in Hailakandi , for the ratio of cases getting essential oils to untreated was 5 to 8 while the ratio of cases getting 'phage on the first day to untreated in Hailakandi after the 17th October was 542 to 33 or 17 to 1

While, therefore, we can say definitely that the 'phage mortality was less than 24 9 per cent the mortality for essential oils was certainly not less than 36 6 per cent

We may, however, compare the effects of the two treatments on cases surviving the first two days of illness, on the deaths from suppression of urine, heart failure, recrudescence of diarrhœa or secondary pneumonia   Here, if treatment is available, the longer a patient lives the more chance has he of getting treatment and of being transferred from the no-treatment class when he was no longer likely to die or when it was too late to help him

By limiting the 'phage cases to those who got 'phage on the first day, we avoid the reception into this group of recovering cases transferred from the untreated groups

<p align="center">TABLE XI</p>

Area	Treatment	Total cases	DEATHS ON 1ST AND 2ND DAYS		DEATHS ON 3RD DAY AND LATER	
			Number	Mortality	Number	Mortality
Silchar	Untreated	804	376	46 8	175	21 8
	Essential oils	481	81	16 8	95	19 8
Hailakandi	Untreated b e f o r e 17th October	121	61	50 4	31	25 6
	Untreated after 16th October	33	26	78 8	2	6 1
	'Phage on first day	542	60	11 07	46	8 49

In Silchar, Table XI, the untreated and the essential oils mortalities were nearly alike after the third day of illness   Among the deaths on the first and second days the untreated deaths preponderate , either treatment with essential oils was useful or the treatment was not being given early enough to include the fulminating cases   Unfortunately in very few cases was the time of giving essential oils noted, though this was asked for in the form

In Hailakandi after the 16th October the mortality in the small untreated group is so high for the first and second days that it may be overladen with fulminating cases and the subsequent mortality may be under-weighted with deaths later. We may, therefore, transfer the whole group to the 'phage class and then the mortality in the 'phage class, weighted with 33 untreated cases and 28 deaths on the first two days, was 16 per cent and 8 5 per cent thereafter as compared with 50 per cent

and 25 per cent among the untreated in Hailakandi and 47 per cent and 22 per cent in Silchar   In nearly all the Hailakandi cases the hour of onset of the disease and the hour when the 'phage was given were recorded, so that 'phage on the first day is definitely 'phage within 24 hours of the onset   'Phage was given on the first day of illness to 542 cases, the mortality was 19 6 per cent   Of these 36 died on the first day and 24 on the second   'Phage treatment began on the second day in 75 cases, these had a mortality of 30 per cent and 10 died on the day treatment began   The days on which death occurred in these two groups are shown in Table XII —

<div align="center">TABLE XII</div>

<div align="center">*Day of illness on which death occurred*</div>

DAY	1	2	3	4	5	6	7	8	9	10	11	12	15	TOTALS
'Phage on 1st day	36	24	18	9	2	4	3	3	1	2	1	1	1	106
'Phage on 2nd day		10	7	2	3	1	0	1	1					25

Comparing the deaths in both groups among those who survive the first two days we have —

<div align="center">TABLE XIII</div>

	Cases	Deaths	Mortality
'Phage on 1st day	482	46	9 5
'Phage on 2nd day	65	15	23 1

This seems to indicate a significant effect from the early administration of 'phage

The last 37 'phage cases in Hailakandi at the tail of the epidemic when the disease was no longer spreading had a mortality of 45 per cent   We noticed this happened in Sibsagar and Darrang in the last few cases   Unfortunately from these cases we did not obtain samples of the stools   It may suggest that mortality and the infectiousness are two attributes of cholera which need not go together

It is possible the explanation of this may be found in laboratory experiments in progress which show that different combinations of 'phage types as distinct from single types have very different actions on the vibrio

Vaccinated persons received 1 c c of anti-cholera vaccine containing 8,000 millions per c c   The 69 persons who were only vaccinated had a mortality of 57 9 and of 14 vaccinated seven days or more before they fell ill 8 died

TABLE

Vaccinated cases

DAYS PREVIOUS TO ILLNESS OF

Days —	0		1		2		3		4		5		6		7		8		9	
Treatment	R	D	R	D	R	D	R	D	R	D	R	D	R	D	R	D	R	D	R	D
Vaccinated only	2	3	3	9	1	9	6	3	7	8	1	0	3	0	1	3	1	1	1	0
Vaccinated and essential oils	14	3	12	5	14	6	14	6	5	1	2	0	3	0	1	2	1	0		
Vaccinated and bacteriophage	4	2	13	2	5	2	7	2	2	1	8	2	4	0	4	1			1	0
Vaccinated, bacteriophage and essential oils																				
TOTALS	20	8	28	16	20	17	27	11	14	10	11	2	10	0	6	6	2	1	2	

R.—Recovered

DAYS ON WHICH DEATH OCCURRED

Days —	1	2	3	4	5	6	7	8	15
Vaccinated only	14	16	2	2	1	2	1	1	
Vaccinated and essential oils	7	9	3	0	2	2			
Vaccinated and bacteriophage	2	4	4	1			1		
Vaccinated, bacteriophage and essential oils					1				

XIV

Cachar district

	10		11		12		14		15		16		17		18		19		20		30		Total cases	Mortality
WHICH VACCINATION WAS DONE	R	D	R	D	R	D	R	D	R	D	R	D	R	D	R	D	R	D	R	D	R	D		
	2	1			1	0	0	1	0	1											0	1	69	57 9
											1	0	2	0									92	25 0
			1	0											1	0	1	0	1	0			64	18 75
	0	1																					1	
	2	2	1	0	1	0	0	1	0	1	1	0	2	0	1	0	1	0	1	0	0	1	226	33 6

D —Died

Two cases excluded   Date of vaccination not known   One died, one recovered

TABLE XV

Mortality with different treatments, Cachar district

	Essential oils	'PHAGE ON 1ST DAY			VACCINATED ONLY			VACCINATED AND ESSENTIAL OILS			VACCINATED AND 'PHAGE			VACCINATED ESSENTIAL OILS AND 'PHAGE
		Observed	Expected	Difference	Observed	Expected	Difference	Observed	Expected	Difference	Observed	Expected	Difference	Observed
Total cases	481	542			69			92			64			1
Deaths on 1st day	44	36	49 6	−13 6	14	6 3	−7 6	7	8 4	−1 4	2	5 9	−3 9	
Deaths on 2nd day	37	24	41 7	−17 7	16	5 3	−10 7	9	7 1	−1 9	4	4 9	−0 9	
Deaths after 2nd day	95	46	107 04	−6 1	10	13 6	−3 6	7	18 2	−11 2	6	12 6	−6 6	1

Contributions to χ^2

'Phage on 1st day	Vaccinated only	Vaccinated and essential oils	Vaccinated and 'phage
3 72	9 41	0 23	2 57
7 51	21 60	0 51	0 17
34 81	0 95	6 89	3 46
46 04	31 96	7 63	[3 20

TABLE XVI

Area	No bacteriophage				Bacteriophage				Total cases	References
	Recovered	Died	Total	Percentage	Recovered	Died	Total	Percentage		
Shillong, 1928	5	16	21	54 3	5	1	6	26 3	27	Annual report of the King Edward VII Memorial Pasteur Institute and Medical Research Institute for 1928
Goalpara, 1928	2	6	8		17	8	25		33	Ditto
North Salmara, 1928	35	28	63		20	6	26		89	Ditto
Jakrem, 1929	18	63	91	77 8	55	7	62	11 0	143	Indian Medical Gazette, **65**, 1930
Sibsagar, 1932	207	102	309	48 1	95	29	124	23 4	523	Annual Report of the King Edward VII Memorial Pasteur Institute and Medical Research Institute for the year 1932
Darrang, 1932	23	115	138	83 3	37	30	67	44 8	205	Ditto
Cachar, 1933	757	944	1,701	55 5	570	152	722	21 05	2,423	
Totals	1,047	1,364	2,411		799	233	1,032		3,443	
Mortality percentage				56 6				22 6		

The no bacteriophage cases include all other treatments as well as untreated cases

The numbers are small but this sample of cases is of interest in that the mortality is only slightly less than the untreated and provides a comparison with other treatments, for these cases were in villages which had been visited before the patient took ill and where others were getting essential oils

In Table XV the various treated cases are compared with what might have been expected had they all had essential oils   In the 'phage group the deaths at each stage of the disease are less

We may compare the mortality with 'phage treatment in this epidemic with previous experiences with 'phage in Assam   For this purpose all cases receiving 'phage at any time before death are included in the 'phage groups, and cases receiving any other treatment with those that had none   The no-'phage groups are representative of the mortality for cholera when measures such as have hitherto been possible in villages are carried out, for at least some of those affected

Sir Leonard Rogers treated 1,003 cases in his ward in Calcutta with hypertonic saline and permanganate of potass with 251 deaths   The mortality was 25 per cent It was the greatest advance in the treatment of cholera up to that time   A further smaller series of 100 cases received atropine in addition and 100 controls had saline and potassium permanganate without atropine   The mortalities were 11 per cent and 22 per cent respectively

These cases had the advantages of a well-equipped hospital and skilled treatment whereas the cases in the Assam villages were in their own huts with no skilled nursing   The days of illness on which the deaths occurred in the Calcutta cases are not given but the causes of death appear under the heads, collapse, uræmia, cardiac failure, pneumonia, hyperpyrexia and sepsis   If we assume that the deaths from collapse happened during the first two days of illness and that the deaths from other causes occurred later, we may compare the Assam results with those of Sir Leonard Rogers and examine the mortality in cases that survived the first two days

TABLE XVII

Place	Treatment	Cases	Deaths	Mortality
Calcutta	Saline and potassium permanganate, 1st series	659	103	15 6
,,	Saline and potassium permanganate, 2nd series	91	14	15 3
,,	Saline, potassium permanganate and atropine	97	8	8 2
Assam	'Phage on 1st day	482	46	9 5
,,	Essential oils	400	95	23 7
,,	Untreated	495	208	42 02

Cases treated with bacteriophage on the first day, and this we have shown can be easily accomplished when bacteriophage is in the hands of the villagers themselves, give results comparable with the best hospital results obtained by Sir Leonard Rogers   Where it is possible to combine 'phage with hypertonic saline for the cases requiring transfusion the results should be better and this is undoubtedly the procedure to be recommended to tide severe cases over the stage of collapse

APPENDIX

Name of village

Date insp	Owner's name with caste and number in family	Patient's name	Age	Sex	Date onset	Treatment and date V—Vaccine B—Bacteriophage	Result, date if died	Previous movements of patient or family feast ?	Specimen taken, number	Position of house in village	General remarks
1	2	3	4	5	6	7	8	9	10	11	12

STUDIES ON THE NUTRITIVE VALUE OF MILK AND MILK PRODUCTS

Part I.

BY

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DRUMMOND, COWARD and WATSON (1921) observed that butter was less potent as a source of vitamin A than the same amount of fat supplied in the original milk from which it was derived and ascribed the differences to the mechanical destruction of a part of the vitamin during the process of churning   Crawford, Golding, Perry and Zilva (1930) working on the fat soluble vitamin of milk came to the conclusion that the entire vitamin A content of milk is associated with milk-fat and that there was no significant loss in the process of separation of fat   They concluded, therefore, that by testing the butter, it should be possible to obtain the approximate relative potency of milk   In view of this contradictory evidence and the need for further knowledge regarding the relative nutritive values of other Indian milk products, such as ghee and curd, the present investigation was undertaken

Ghee is the most common fat which forms an important item in diet throughout India   The vitamin A content of ghee was first studied by Ghosh (1922), who observed that several samples of Indian ghee had a vitamin content as good as butter but his results were obtained when vitamin A had not been distinguished from vitamin D   Bacharach (1930) obtained three samples of ghee from Bombay which were selected as typical of those available in the open market and found that only one out of three samples showed a measurable blue colour with antimony trichloride   On feeding rats with the sample which responded to the colour test, he found that 5 g of cod-liver oil was markedly superior to 200 times the quantity of ghee   He remarked that for practical purposes the ghee must be devoid of vitamin A and suggested that the problem needs further and official investigation

Brahmachari (1932) observed that rats, receiving a supplement of ghee to the extent of 16 per cent of the basal diet, died after 4 weeks   Unfortunately, Brahmachari could employ only two rats for his experiment   It is quite likely, however, that the ghee and butter obtained from the market are mostly adulterated and, therefore, lacking in vitamin A   We carried out feeding experiments, therefore, with the object of finding out the extent to which the growth promoting factor of pure ghee compares with that of the same quantity of original butter

Experimental

Young rats of an age of 28 to 30 days weighing 35 g to 40 g were placed on a vitamin A free basal diet consisting of—

Purified rice starch	71 parts
Casein (B D H)	20 ,,
Salt mixture (McCollum)	4 ,,
Dried brewer's yeast	5 ,,

In the first series of experiments vitamin D was introduced into the ration by irradiation of the food with a mercury vapour quartz lamp at a distance of 28 inches for a period of fifteen minutes   During subsequent experiments the antirachitic factor was supplied in the form of Radiostol, each animal receiving one drop twice a week during the preparatory and test periods

In the first experiment the morning milk of a Scindi cow aged 11 years, kept at the Imperial Institute of Dairying and Animal Husbandry, Bangalore, was used The cow was given a measured quantity of ration throughout the course of the experiment   The average yield of milk per day was 17 lb   The milk yield record at Bangalore dairy of the Scindi cow is given below —

Lactation	Milk yield, lb	Days on milk	Days dry	Fat, per cent	Total solid not fat
1	1,370	154	287	3 5	8 85
2	4,294	257	91	4 0	8 61

Butter making was carried out at the dairy, through the kindness of the Imperial Dairy Expert and under the immediate supervision of Mr Cox, the Dairy Superintendent—to both of whom our thanks are due   Ghee was prepared in the laboratory by melting the butter and heating till it gave off the characteristic odour of ghee   Ghee and butter were preserved in the cold room throughout the course of the experiment   From 200 g of butter 160 g of ghee were obtained   After the depletion period, the different groups of rats were fed with basal diet supplemented with a liberal supply of milk (20 c c) and a corresponding quantity of ghee and butter

TABLE I

Growth chart of individual rats on basal diet with supplements of milk, butter and ghee (milk used from Scindi cow No I)

Supplement	Rat number	Sex	Initial weight, g	Final weight, g	Days under experiment	Total increase in weight, g	Increase in weight per week, g
Milk, 20 c c	1	♂	83	169	30	86	20 0
	2	♂	74	160	30	86	20 0
	3	♀	64	130	30	66	15 4
	4	♀	70	146	30	76	17 7
						Average	18 2
Milk autoclaved for 15 mins at 15 lb, 20 c c	5	♂	78	151	30	73	17 0
	6	♂	67	142	30	75	17 5
	7	♀	73	139	30	66	15 4
	8	♀	72	132	30	60	15 0
						Average	16 2
Butter, 1 g	9	♂	86	140	30	54	12 6
	10	♂	73	121	30	48	11 2
	11	♀	68	112	30	44	10 2
	12	♀	66	106	30	40	9 3
						Average	10 8
Ghee, 0 8 g	13	♂	68	119	30	51	11 9
	14	♂	80	124	30	44	10 2
	15	♀	70	116	30	46	10 7
	16	♀	66	113	30	47	10 9
						Average	10 9

TABLE II

*Growth chart of individual rats on basal diet with supplements of milk,
curd, butter and ghee (milk used from a common village cow,
daily yield of milk only 4 lb., fat 5 per cent)*

Supplement	Rat number	Sex	Initial weight, g	Final weight, g	Days under experiment	Total increase in weight, g	Increase in weight per week, g
Milk, 20 c c	17	♂	91	157	30	66	15 4
	18	♂	83	152	30	69	16 1
	19	♂	70	138	30	68	15 5
	20	♀	98	156	30	58	13 5
	21	♀	78	126	30	48	11 0
						Average	14 3
Curd, 20 c c	22	♂	89	147	30	58	13 5
	23	♂	80	142	30	62	14 4
	24	♀	80	132	30	52	12 1
	25	♀	82	134	30	52	12 1
						Average	13 0
Butter, 1 2 g	26	♂	83	123	30	40	9 3
	27	♀	99	135	30	36	8 4
						Average	8 8
Ghee, 1 g	28	♂	83	124	30	41	9 5
	29	♂	90	130	30	40	9 3
	30	♀	96	131	30	35	8 1
	31	♀	65	101	30	36	8 4
						Average	8 8
Skimmed milk, 20 c c , plus butter, 1 2 g	32	♂	83	151	30	68	15 8
	33	♀	83	139	30	56	13 0
	34	♂	90	156	30	66	15 4
						Average	14 7

In the third set, the feeding experiment was carried out with the minimum quantity of milk (4 c c) and corresponding quantities of butter and ghee   The milk from cow No II was used   A preliminary study showed that with still lower levels, viz , 2 c c and 3 c c of milk, proper and consistent growth was not obtained
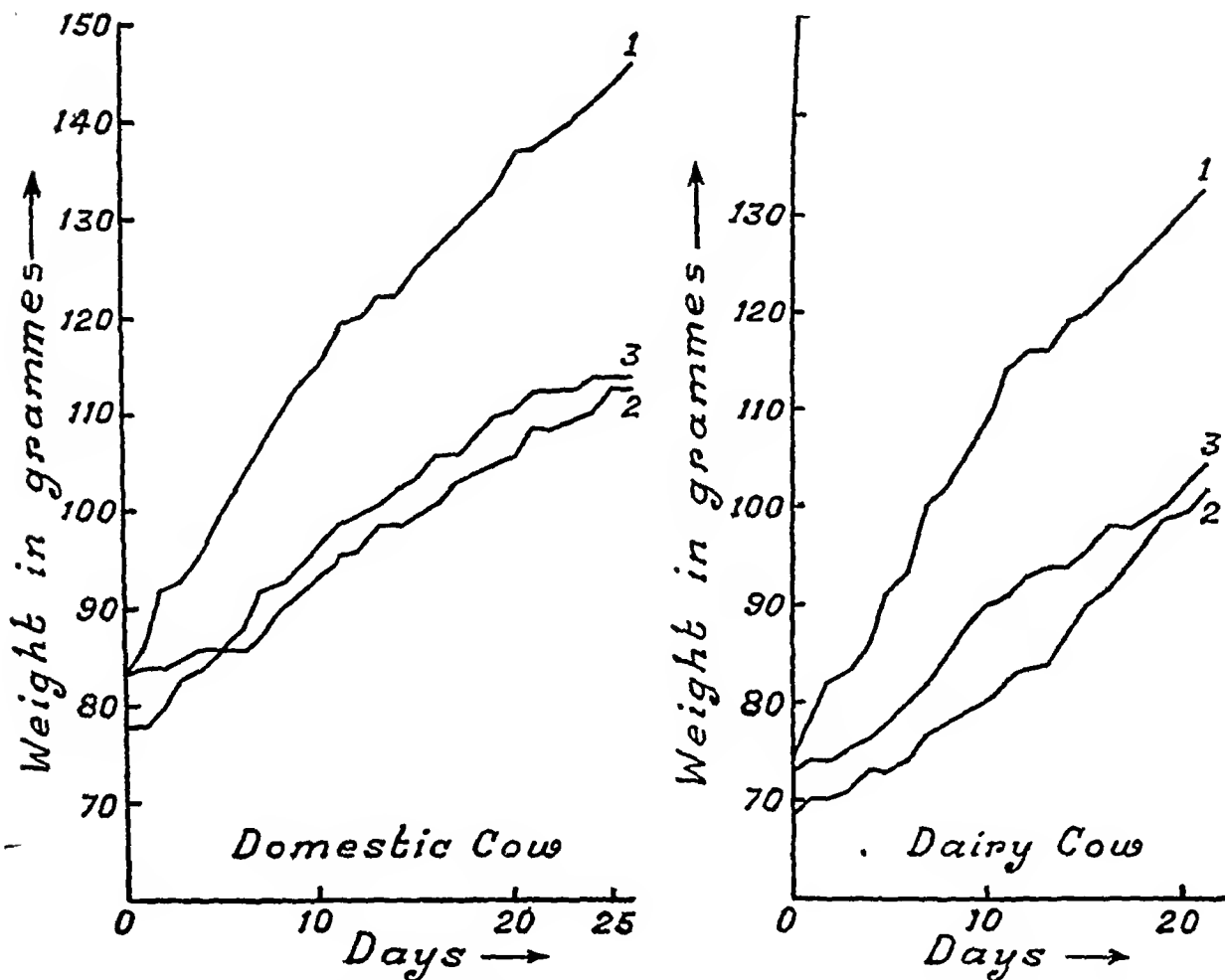
Fig 1   Growth curve of rats maintained on basal diet supplemented with (1) milk, 20 c c , (2) ghee, (3) butter (Ghee and butter corresponding to 20 c c of milk fat)

The rats maintained on 20 c c of milk showed the maximum growth, whereas those rats with the supplements of butter and ghee had a growth rate of only 56 to 60 per cent of those on original milk (Tables I and II)   Twenty c c of skimmed milk together with the separated fat maintained growth in rats equal to original

milk showing that there was no loss of vitamin A in the process of separation of fat

TABLE III

Growth chart of individual rats with the minimum quantity of milk, ghee and butter

Supplement	Rat number	Sex	Initial weight, g	Final weight, g	Days under experiment	Total increase in weight, g	Increase in weight per week, g
Milk, 4 c c	35	♂	97	122	30	25	5 8
	36	♂	86	113	30	27	6 3
	37	♀	97	122	30	25	5 8
						Average	6 0
Butter, 0 25 g	38	♂	94	124	30	30	7 0
	39	♂	98	126	30	28	6 5
	40	♀	84	105	30	21	4 9
	41	♀	80	102	30	22	5 1
						Average	5 8
Ghee, 0 2 g	42	♂	83	109	30	26	6 0
	43	♂	77	103	30	26	6 0
	44	♀	82	104	30	22	5 1
	45	♀	90	113	30	23	5 3
						Average	5 6

The results of the feeding experiment (Table III) with minimum quantities of milk, butter and ghee show that the growth was the same in all cases, indicating that the entire vitamin A content of milk is associated with milk-fat as observed by Zilva and his co-workers (Crawford *et al*, *loc cit*)

The apparent discrepancy between the results of the two sets of experiments can be explained by supposing that when the animals were fed with supplements of milk, butter or ghee at a lower level, the vitamin B in the yeast supplied along with the basal diet was sufficient for the lower rate of growth as required by the minimum

quantity of vitamin A present in milk-fat   Hence equal rate of growth was obtained in each case (Fig 2)   But when the animals were given milk-fat at a higher level (viz , 1 g of milk-fat either as butter or ghee), the growth rate was much lower compared to original milk   It is possible that in the case of original milk, the excess of water soluble vitamin B or some additional factor in milk along with yeast was responsible for increased growth
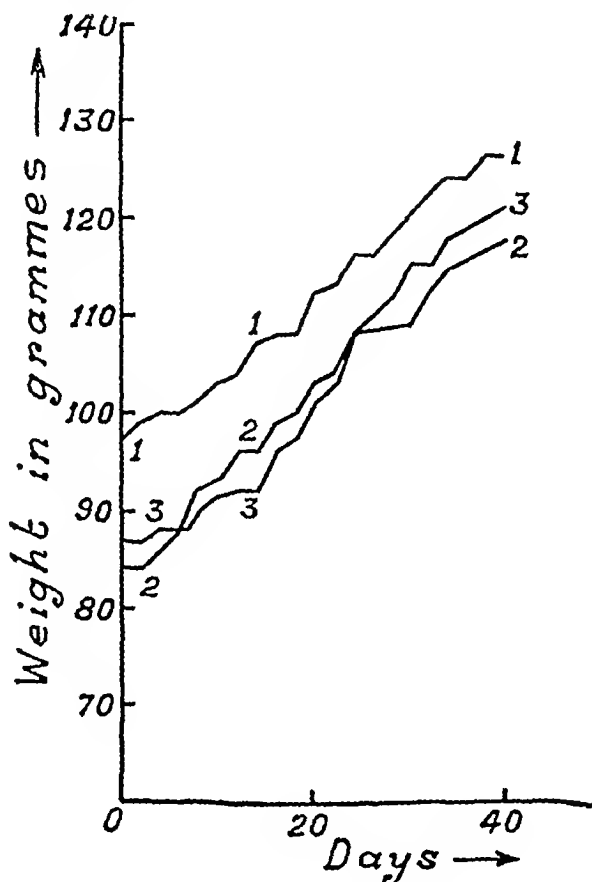
Fig 2   Growth curve of rats maintained on basal diet
supplemented with (1) milk, 4 c c , (2) ghee, (3) butter
(Ghee and butter corresponding to 4 c o of milk fat)

It was observed that addition of Marmite along with butter and ghee failed to produce better growth but the addition of skimmed milk together with butter induced growth in rats equal to original milk

Claire, Graham and their co-workers (1933) have also observed that 0 5 g of yeast supplemented with increasing quantities of cod-liver oil, viz , 0 09, 0 27, 0 36 g , failed to produce better growth than 0 09 g of cod-liver oil   Increasing the quantity

of yeast failed to induce increased growth to a great extent   But the addition of liver to a smaller quantity of yeast resulted in improved growth   These suggest the possibility that there is some other factor beside vitamins B_1 and B_2 present in the water soluble fraction of milk and also in the liver as suggested by Guha (1931)

Butter and ghee—The result of feeding experiments with rats using supplements of butter and ghee along with the basal diet show that the nutritive value of pure ghee was as good as that of original butter   The same conclusion has been reached by feeding rats with supplements of butter and ghee both at a higher and also at a lower level   Butter does not seem to lose its nutritive value in the process of preparation of ghee   Butter-fat is exceptional in this respect   Hopkins found that fat soluble vitamin A in butter displays marked resistance to heat but destruction occurs on heating to a high temperature of 120°C by simultaneous aeration of the fat

The effect of milk on the rate of growth—Although the known facts regarding the nutritive requirements of men and animals have increased during the last twenty years, it is difficult to state definitely the nature of combinations of food necessary for proper growth   Since it has been shown by Guha (*loc cit*) that milk exerts a stimulating effect upon the growth of rats during the period when they cease to grow when kept on a basal diet supplemented with cod-liver oil and yeast, attempts have been made to study the effect of addition of milk as a supplement to some of the natural food-stuffs in common use

Three different combinations of food were tested   Different groups of rats weighing about 50 g were maintained on the following composition of food, so that all the groups of rats were supplied with vitamins A and B in some form or other —

(1) Rice, dhal, cooked vegetables including carrots
(2) Rice and dhal with supplement of cod-liver oil and Marmite
(3) Wheat chappati and milk (20 c c)

TABLE IV

Group	Rat number	Sex	Original weight, g	Final weight range, g	Average increase in weight, g	Days to reach 200 g (males only)
			RESULTS FOR FIRST 60 DAYS			
I	5	♂	56	140–143	85	
II	5	♂	55	150–170	101	96
III	5	♂	54	175–189	128	77
I	4	♀	56	105–113	53	
II	4	♀	52	124–139	80	
III	5	♀	51	128–146	80	

The effect of milk in accelerating the rate of growth is clearly shown by rats of group III (Table IV) which gives the growth rate for 60 days (the period of most rapid growth) and also the number of days required to reach 200 g   The rats of this group showed a steady rate of growth all through (Fig 3, curve 3) and was the
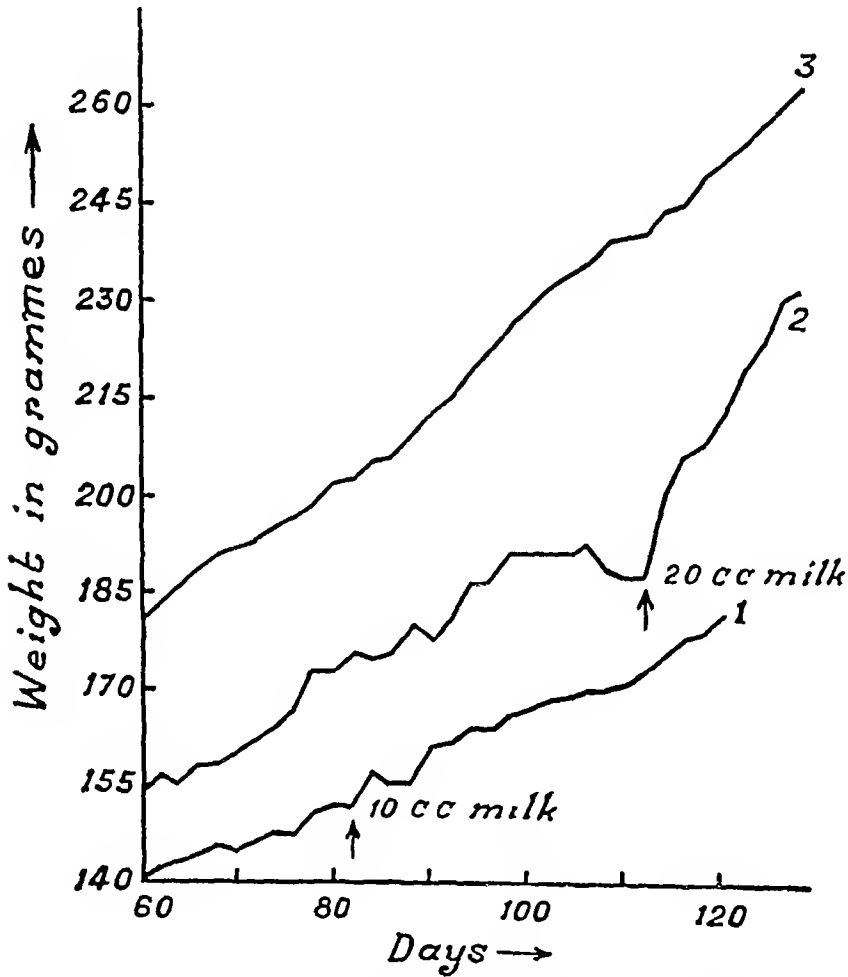
Fig 3   Growth curve of male rats maintained on (1) rice, dhal and vegetables including carrots, (2) rice, dhal with supplements of cod liver oil and Marmite, (3) wheat chappati and 20 c c of milk

only group to reach 300 g which is nearly the optimum growth   The rats of group II with cod-liver oil and Marmite had their growth rate very near to those of group III during 80 days, after which it became slower and slower till they reached 200 g to 210 g   The change of supplement from cod-liver oil to milk had a marked effect

on growth (Fig 3, curve 2) The animals of group I had a poor rate of growth and reached only 140 g to 150 g Addition of 10 c c of milk slightly improved the growth A pronounced stimulating effect of milk on growth of rats was observed in each case Although vegetables like cabbage and carrots contain vitamins A and B, they cannot maintain optimum growth in male rats *A liberal supply of milk is essential along with a vegetarian diet for proper growth during the growing period*
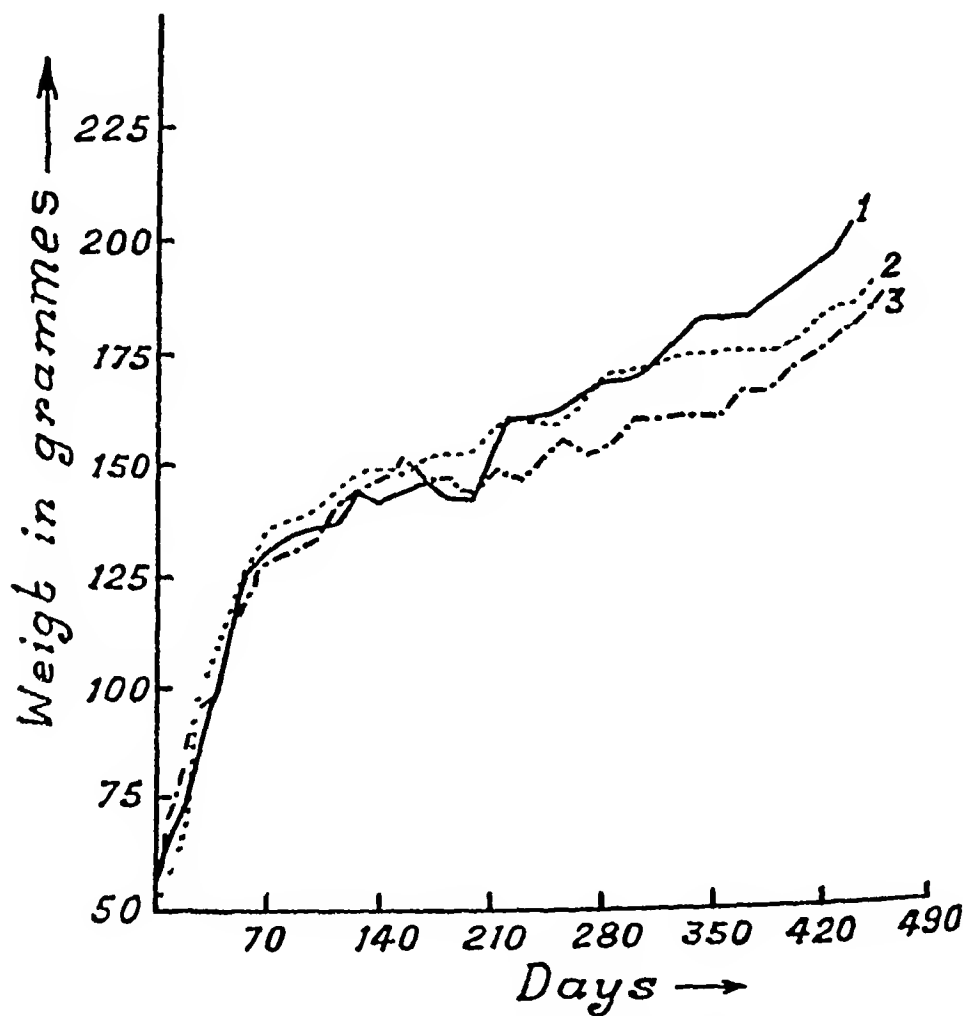
Fig 4 Growth curve of female rats maintained on wheat chappati supplemented with (1) butter, (2) skimmed milk, (3) cod liver oil and Marmite

Behaviour of female rats —The results of feeding experiments on female rats (group II) using supplements of cod-liver oil and Marmite show that the female rats grow steadily for a period of 60 to 70 days till they reach 135 g to 145 g , but subsequently they show very poor growth Changing the supplement to 20 c c of milk failed to improve growth The rats of group III getting wheat chappati and 20 c c of milk behaved in the same way Similar results were also obtained by Guha

(*loc cit*) using basal diet supplemented with 15 c c to 20 c c of milk   According to him the new factor present in milk exhibits a more marked stimulating effect on the growth of male rats than in that of females   We extended the period of feeding experiments for one and a half years using wheat chappati and different supplements like butter-fat, cod-liver oil and skimmed milk   As can be seen from Fig 4, rats of all groups had continuous growth for 70 days after which they showed very slight growth for a period of four to five months and finally they again began to grow better till they reach 250 g to 300 g   The females seem to have three distinct phases in the period of growth   It is possible that during the second stage (the period of little or no growth), which covers about four to five months just after the time when they become mature, they reserve all the vitamins for their litters

SUMMARY

1   The growth promoting factor in the milk of a Scindi cow (stall fed) kept in the Dairy Farm is slightly higher than that of an ordinary cow (pasture cow), in spite of the fact that the daily yield of a dairy cow is much higher

2   The nutritive value of pure ghee is found to be the same as that of the original butter

3   The growth promoting factor of curd is found to be very nearly the same as that of the equivalent quantity of milk

4   Female rats showed little or no growth for a period of four to five months just after their maturity, even with supplements of sufficient milk, butter or cod-liver oil

5   For practical purposes, the nutritive value of milk is superior to that of butter or ghee   Milk possesses a pronounced stimulating effect on growth and hence greater consumption of milk should be advocated

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STUDIES IN LIPOID METABOLISM

Part I

VARIATION IN CHOLESTEROL CONTENT OF BLOOD AND OF DIFFERENT ORGANS IN PIGEONS CONSEQUENT ON ADMINISTRATION OF CHLOROFORM

BY

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ALTHOUGH cholesterol forms an important constituent of all living cells, its physiological function in the living organism is not yet properly understood   The variation of blood cholesterol under the action of aliphatic narcotics like ether and chloroform has been studied by several workers with the hope of obtaining evidence regarding its rôle in the animal organism   Ducchesch (1919) administered chloroform to dogs for from 1 to $1\frac{1}{2}$ hours daily for several days and observed that serum cholesterol increased in moderate degrees during two or three days of narcosis and then went down to low values   Mahler (1926) studied the cases operated on, in the Lebanon Hospital, New York, and observed that under ether anæsthesia in man, the cholesterol increases in blood but if insulin is administered before anæsthesia, there is no increase in blood cholesterol   Gray (1930) observed a definite rise in blood cholesterol, beginning after three weeks, on repeated administration of chloroform to rabbits   The observation of Gray did not agree with those of Ducchesch , the former explained it as being due to differences in the animals used In order to get definite information as to the real significance of the variation in blood cholesterol, the present investigation was undertaken

The following is an outline of the series of experiments conducted the effect of repeated inhalation of chloroform in pigeons on (1) the cholesterol content of blood, (2) the cholesterol content of different tissues, (3) the relation between cholesterol, and the number of red cells in the blood

EXPERIMENTAL

The pigeons were subjected to chloroform inhalation by putting them in a box which contained cotton-wool saturated with chloroform   The birds were kept in an atmosphere of chloroform for from $1\frac{1}{2}$ to 2 hours daily before giving them food

(353)

Effect of chloroform on the cholesterol content of different tissues —For the estima tion of cholesterol in different tissues such as brain, lungs, liver, kidney and heart, the pigeons were killed and the gross fat and adhering blood were removed from the tissues The different tissues were thoroughy powdered with anhydrous sodium sulphate and subjected to exhaustive extraction with chloroform Since chloro form does not extract all the lipoids, the residue was again extracted with alcohol and the alcohol was removed by distillation at a low temperature in an atmosphere of carbon dioxide The dried residue was treated several times by boiling with fresh quantities of chloroform The combined chloroform extract was kept for analysis

Estimation of total chloroform extract —An aliquot portion of the chloroform extract of the different tissues was taken in a weighing bottle, the chloroform was removed on a water-bath at 50°C to 55°C in an atmosphere of carbon dioxide The bottle was dried in a desiccator and weighed

Estimation of free cholesterol —The free cholesterol was estimated gravimetrically as cholesterol digitonide The technique employed by Jowett and Lawson (1931) was followed

Estimation of total cholesterol —The dried chloroform extract was dissolved by warming with 1 c c of ether and saponified with 15 per cent sodium ethoxide in absolute alcohol by allowing to stand for 24 hours (Gardner and Fox, 1924) The solution was dried, 2 c c of 2N sulphuric acid added and extracted with dry ether The cholesterol digitonide was precipitated in ethereal solution In the case of brain and lungs colourimetric methods also gave good results with the original solu tion

TABLE IV

Cholesterol content of different organs

Brain

Pigeon number	Weight of brain (g)	Total choles- terol (mg)	Free choles terol (mg)	Weight of chloroform extract (g)	Total choles terol percent- age of brain	Free choles terol percent age of brain	Chloroform extract percentage of brain
Control—							
4	1 796	26 5	25 6	0 149	1 47	1 42	8 29
5	1 945	28 5	28 5	0 150	1 46	1 46	7 66
9	1 790	26 0	25 1	0 137	1 45	1 45	7 86
			Average		1 46	1 42	7 86

N C Datta

Brain—concld

Pigeon number	Weight of brain (g)	Total cholesterol (mg)	Free cholesterol (mg)	Weight of chloroform extract (g)	Total cholesterol percentage of brain	Free cholesterol percentage of brain	Chloroform extract percentage of brain
Experimental—							
1	1 750	25 0	23 8	0 134	1 42	1 36	7 76
2	1 600	23 5	21 7	0 127	1 46	1 35	7 93
6	2 040	30 0	30 0	0 157	1 46	1 46	7 67
7	2 070	29 0	28 5	0 161	1 10	1 37	7 77
3	1 700	25 2	25 0	0 143	1 48	1 47	8 41
Average					1 44	1 40	7 88

The cholesterol content of brain was all in the free state There was practically no difference between the control and the experimental pigeons as regards their cholesterol content and the total chloroform extract of the brain

Liver

Pigeon number	Weight of liver (g)	Total cholesterol (mg)	Free cholesterol (mg)	Weight of chloroform extract (g)	Total cholesterol percentage of liver	Free cholesterol percentage of liver	Chloroform extract percentage of liver
Control—							
5	7 931	31 5	22 9	0 345	0 397	0 288	4 34
8	6 941	23 3	18 7	0 545	0 335	0 269	7 85
9	4 895	17 5	12 8	0 285	0 357	0 261	5 82
Average	6 580				0 363	0 272	6 00
Experimental—							
1	8 251	25 1	20 0	0 417	0 304	0 242	5 04
3	9 011	30 0	20 9	0 359	0 332	0 232	3 98
7	8 210	28 5	20 7	0 382	0 347	0 252	3 98
6	6 203	19 4	14 3		0 311	0 231	4 33
Average	7 910				0 323	0 239	3 98

Studies in Lipoid Metabolism

A slight difference was observed between the control and the experimental pigeons as regards the total cholesterol content and total chloroform extract of the liver The difference was not of such an order as to explain the increase in blood cholesterol As a result of prolonged inhalation of chloroform, the liver was found enlarged with the result that the weight of the liver was slightly increased This might be the reason for the small difference in the total cholesterol content between the control and the experimental pigeons

Lungs

Pigeon number	Weight of lungs (g)	Total choles terol (mg)	Free choles terol (mg)	Total chloroform extract (g)	Total choles terol percent age of lungs	Free choles terol percent age of lungs	Chloroform extract percentage of lungs
Control—							
4	1 941	13 25	13 00	0 075	0 682	0 669	3 86
8	2 806	17 27	16 70	0 116	0 615	0 602	4 13
9	2 574	16 00	14 00	0 085	0 621	0 543	3 30
				Average	0 639	0 604	3 76
Experimental—							
7	2 402	16 25	14 58	0 072	0 676	0 607	3 00
1	2 750	17 60	16 40	0 086	0 640	0 595	3 14
3	3 411	21 30		0 112	0 624		3 28
				Average	0 646	0 601	3 14

The cholesterol is present mostly in the free state in the lungs   The inhala tion of chloroform has no effect on the cholesterol content of the lungs

Kidney

Pigeon number	Weight of kidney (g)	Total choles terol (mg)	Free choles terol (mg)	Total chloroform extract (g)	Total choles terol percentage of kidney	Free choles terol percentage of kidney	Chloroform extract percentage of kidney
Control—							
5	1 607	6 35	5 98	0 059	0 395	0 372	3 67
8	1 735	7 03	6 60		0 405	0 380	
9	1 988	6 80	6 50	0 080	0 342	0 326	4 04
				Average	0 380	0 359	3 85
Experimental—							
7	1 632	7 00	5 60	0 068	0 428	0 343	4 20
6	1 685	6 50	5 98	0 059	0 383	0 353	3 52
3	2 410	8 70		0 085	0 360		3 52
				Average	0 390	0 348	3 75

Inhalation of chloroform had no effect either on the total or the free cholesterol content of the kidney

Heart

Pigeon number	Weight of heart (g)	Total choles terol (mg)	Free choles terol (mg)	Total chloroform extract (g)	Total choles terol percentage of heart	Free choles terol percentage of heart	Chloroform extract percentage of heart
Control—							
5	2 934	5 30	3 79	0 228	0 180	0 129	7 70
8	2 646	4 36	3 95	0 303	0 172	0 149	11 20
9	2 968	5 30	4 30	0 334	0 168	0 144	11 20
				Average	0 170	0 140	10 00
Experimental—							
2	2 871	4 00	3 35	0 282	0 139	0 116	9 80
6	2 896	5 20	4 40	0 118	0 179	0 151	4 00
7	2 945	5 00	4 37	0 103	0 168	0 148	3 50
				Average	0 162	0 138	5 80

The cholesterol content of the heart was found to be quite normal   A marked decrease in the chloroform extract of the heart was observed in the experimental pigeons.

Adipose tissue

Pigeon number	Weight of adipose tissue	Total choles-terol (mg)
Control—		
5	3 618	25 40
8	3 800	52 30
9	3 760	25 00
4	3 132	11 50
Experimental—	*Nil*	*Nil*

In the case of experimental pigeons there was no fat under the subcutaneous tissues or in the region of muscles or liver   In only one case, an insignificant amount of fat was observed

Relation between cholesterol and R B C —Many workers in the past have tried to establish a relationship between cholesterol and the number of red cells in blood Csonka (1916) found that in pernicious anæmia the cholesterol content of blood was below the normal   Denis (1917) obtained a subnormal value for cholesterol in blood in anæmia but no definite relationship was found to exist between cholesterol and the number of red cells   Muller (*loc cit*) fed pigeons with different organs of the cow after a period of fasting that rendered them anæmic and observed that the greatest cholesterol increase in blood appeared after cow's liver had been fed them, while when fed with spleen and heart the cholesterol content was found to be lowest By observing the variation in number of red cells in the blood, he found that liver was the least and spleen and heart were the most effective as regards regeneration of red cells   He concluded that there was a striking inverse relation between the number of red cells and the increase in blood cholesterol   Castex *et al* (1931) observed that following cisternal puncture, the blood cholesterol was lowered in 13 out of 16 patients   Cisternal puncture causes a diminution in the number of red cells   In 5 cases a distinct parallelism was observed between the variation in number of erythrocytes and the cholesterol content   The variation of blood cholesterol and the number of red cells in the blood of pigeons under the action of prolonged chloroform inhalation was studied by me with the hope of obtaining some relation-ship between the two

TABLE V

Variation of blood cholesterol and R B C in blood of pigeons

CONTROL PIGEON No 4			CONTROL PIGEON No 8		
Date	Cholesterol per 100 c c of blood (mg)	R B C in millions per c mm	Date	Cholesterol per 100 c c of blood (mg)	R B C in millions per c mm
October 15	220	4 04	January 15	225	4 28
November 5	215	4 04	„ 27	228	4 32
„ 19	215	4 04	February 15	200	4 08
December 8	210	4 08	March 1	200	3 80
„ 18	215	4 00	„ 10	190	3 80
January 2	210	4 00			

EXPERIMENTAL PIGEON No 2			EXPERIMENTAL PIGEON No 3		
Date	Cholesterol per 100 c c of blood (mg)	R B C in millions per c mm	Date	Cholesterol per 100 c c of blood (mg)	R B C in millions per c mm
October 15	240	4 00	September 30	192	3 76
November 5	260	4 20	October 12	220	4 00
„ 19	260	4 20	„ 26	260	4 28
December 8	285	4 20	November 10	250	3 92
„ 18	288	4 24	„ 27	255 Fall	3 96
January 2	290	4 00	December 15	235	3 52
			January 1	205	3 36

In the case of control pigeon No 4, both the cholesterol and red blood cells in blood remained almost constant, whereas in that of pigeon No 8, there was a tendency towards diminution in the number of red blood cells with the lowering of cholesterol in blood   In experimental pigeons there was a slight increase in the

number of red cells during the course of increase in blood cholesterol as in the case of pigeon No 2   But if the period of chloroform inhalation was extended further, a marked fall in the number of red cells was observed along with the lowering of blood cholesterol as instanced by pigeon No 3

<div align="center">DISCUSSION</div>

It is clear from Table IV that cholesterol present in the brain is all in the free state   There was practically no difference between the control and the experimental pigeons as regards the percentage of total chloroform extract and the cholesterol content of the brain   Hence, the rise of blood cholesterol was not due to the dissolving out of brain lipoids as observed by Bibra and Harless (quoted by Bloor, 1914) who found that the ether soluble material of the brain was diminished under ether anæsthesia   A slight difference in the percentage of total cholesterol in the liver was observed, but the variation was of a very small order   The cholesterol content of the other tissues, in the experimental pigeons, was found to be quite normal The cholesterol of the different tissues, which is mostly in the free state, seems to be very resistant and unaffected by the action of a drug like chloroform   A marked difference was observed between the control and the experimental pigeons as regards the fat and the cholesterol of the adipose tissues, the reserve fat in the experimental pigeons being completely used up under the influence of chloroform   It is probable, therefore, that the reason for the excess of cholesterol in blood due to chloroform inhalation may be ascribed to mobilization of reserve fat in the blood rather than to any destruction of tissues

The way in which the reserve fat is put into circulation is very little understood, but the fact that increase in blood cholesterol is always associated with a decrease in body-weight suggests the possibility that besides simple mobilization of fat, some other metabolic disturbance also takes place in the system   Ross and Davis (1920) observed that hyperglycæmia in dogs was due to a direct suppression of the internal secretion of the pancreas by ether   Bloor (1916) observed that in diabetes mellitus, the hyperglycæmia was accompanied by hypercholesterolemia It is probable that under the action of prolonged chloroform inhalation, the internal secretion of the pancreas is partially stopped and a condition is set up in the system similar to that observed in diabetes mellitus   In the absence of this secretion, the rate of carbohydrate combustion is reduced, fat is mobilized from the reserve to meet the energy requirements of the body   Acetonuria or acetone and ketonic bodies which are found in diabetic urine were also observed by Baldwin (1905) following chloroform inhalation   The tissue protein is broken down to supply the anti-ketogenic fuel resulting in loss of body-weight   Hence, increase in cholesterol in blood is an indication of a great demand for fat metabolism, as suggested by Campbell (1925) in the case of starvation   But when the reserve fat is used up under the action of chloroform, the cholesterol was found to come down to a normal value again, and an organ like the heart, which requires the greatest energy for its working, acts upon its own tissue-fat as shown by the distinct lower value of chloroform extract of the heart in experimental pigeons (*see* Table IV)

An increase in the lipoid constituent of the blood occurs in the case of diabetes mellitus and, in fact, Rabinowitch (1927) reported from observation on the cholesterol value of large numbers of patients that determination of blood lipoid affords

a better measure of severity of disease than determination of blood sugar   According to Peters and van Slyke (1931) determination of cholesterol will give an index of severity of the diabetic condition only in untreated cases   But even in untreated conditions, unless the variation of blood cholesterol is followed from time to time, the determination of blood cholesterol at a single point may not give a true indication of conditions   Wilson (1907) observed that in serious cases, when all the body-fat is used up, the blood did not show conditions of lipæmia   Hence, if the increase in blood cholesterol is due to mobilization in the blood, of reserve fat in serious cases when all the body-fat is used up, the blood cholesterol after the period of increase will again come down to normal values   Since it has been observed (Table V) that under the action of chloroform on pigeons the number of red cells remains practically constant during the period of increase in blood cholesterol, but shows a marked decrease with lowering of cholesterol, determination of the number of red cells (R B C) along with cholesterol may give an index of the severity and the progress of the disease in diabetes mellitus   Further work on these lines with diabetic blood is in progress

SUMMARY

1   Repeated inhalation of chloroform in pigeons produced an increase in blood cholesterol during the first two months and then went down to low values again

2   Increase in blood cholesterol in pigeons under chloroform was associated with a decrease in body-weight

3   Inhalation of chloroform had practically no effect on the cholesterol content of brain, lungs, heart and kidney , the liver was found to be slightly affected   A marked difference was, however, observed between the control and the experimental pigeons as regards adipose tissue-fat

4   No definite relationship was found to exist between the cholesterol and the number of red cells in blood   During the period of increase in blood cholesterol under chloroform, the number of red cells remained almost constant but there was considerable decrease in the number of red cells along with lowering of blood cholesterol

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CONCENTRATION OF ANTIVENENE BY THE AMMONIUM SULPHATE METHOD

BY

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AFTER Calmette had prepared a polyvalent antivenene at the Pasteur Institute , Lille, the preparation of a similar antiserum was commenced at the Pasteur Institute of India, Kasauli, in 1901 (Sanitary Commissioner with the Government of India, 1901) By 1902 ' 1,020 bottles, each containing 15 c c , were distributed all over India ' (Sanitary Commissioner with the Government of India, 1902) It was soon realized that excepting the venom of the cobra (*Naia*), which produces an antivenene active against the venoms of the various species of the genus, the different snake venoms produced antivenenes which were strictly specific , hence the necessity in India of preparing an antivenene against the Russell's viper and the cobra, two of the commonest causes of death from snake-bite

In 1905 the Central Research Institute, Kasauli, took over the preparation of the antivenene from the Pasteur Institute (Sanitary Commissioner with the Government of India, 1906) and started issuing to the public a combined antivenene against the cobra (*Naia*) and the viper (*Vipera russelli*) During the following 29 years the details of preparation and standardization of the serum underwent very little change, and it was not until this year that a concentrated antivenene was prepared and issued to the public

Considerable experimental work in this connection had been previously done by Acton and Knowles (1915), Caius, Iyengar and Anderson (1924) and Mallick and Maitra (1932) Maitra, Naidu and Ahuja (1933) by using a sodium sulphate method actually produced a concentrated antivenene suitable for issue

In the present communication an application to antivenene of the standard method of concentration of antisera by ammonium sulphate is described A comparison is also made with the sodium sulphate method previously described in this *Journal* (Maitra, Naidu and Ahuja *loc cit*)

THE TECHNIQUE

To the plasma of immunized horses (obtained by adding to the blood 1 per cent of a 10 per cent solution of sodium oxalate and preserved by the

addition of 0 7 per cent of a mixture of equal volumes of trikresol and ether) are added (*a*) 3 per cent of a 4 per cent solution of calcium chloride, (*b*) two volumes of tap-water and (*c*) of the total volume so obtained about 18 per cent of ammonium sulphate   The specific gravity is adjusted to 1098 (should not be *above* 1098, may be 1097) by adding more of the salt or tap-water   After half an hour the specific gravity is found to be about one degree higher   It is finally adjusted to 1099 (should not be *above* 1099, may be 1098) and the mixture filtered through chain cloth folded like a concertina along the border to fit a funnel   The filtrate is returned to the filtering cloth until it comes through clear   The precipitate on the cloth represents the fibrin and the euglobulin   In the clear filtrate pass the pseudoglobulin and the albumen   The precipitate is rejected

To the filtrate after measurement is added about 10 per cent more of the ammonium sulphate and the specific gravity adjusted to 1133 (should not be *below* 1133, may be 1134)   The mixture is stirred, allowed to stand for half an hour, stirred again and filtered through another piece of chain cloth   The precipitate collected on the cloth represents the pseudoglobulin   In the filtrate passes out the albumen   The filtrate is rejected

The precipitate is scraped towards the middle of the cloth with an ivory spatula The cloth is folded in such a way over the precipitate as to leave no folds and dead spaces   It is then subjected to pressure in a hand press   The pressure is applied at first gradually then increased quickly until dripping ceases   The hard-pressed precipitate is removed from the cloth as a cake

In the first filtration (for the removal of the fibrin and the euglobulin) as much of the filtrate is collected as possible   It is an advantage to use a *round* piece of chain cloth to reduce the absorption by the cloth to a minimum   Further, small quantities of an 18 per cent solution of ammonium sulphate (specific gravity 1099) are poured on the filtering surface near the border until the drippings are free from colour   This additional quantity of filtrate is added to that first collected

After the second filtration (for the collection of the pseudoglobulin) the precipitate is pressed as hard as possible   For this purpose a square piece of chain cloth is of advantage

The cake is weighed, broken up and put in bags of cellophane (No 300), in quantities of about 100 grammes for each   The bags are suspended from a rod and allowed to dip in running cold water

In 12 hours the contents of the bags are found to be liquid, clear and increased in bulk   The bags are now gently squashed and allowed to sink further, to increase the surface for dialysis   In 24 hours the contents begin to turn turbid   The turbidity increases steadily and is at a maximum in 96 hours   By this time the ammonium sulphate has dialysed out

To test the completion of the dialysis the flow of water is stopped for 15 minutes and the film of water on the bags is collected by lifting them into a Petri dish   The film-water is matched against the tap-water after adding to both, in two test-tubes, a few drops of a solution of barium chloride   A perfect matching of the turbidity shows that, for all practical purposes, the ammonium sulphate has been removed

The dialysate is collected into a bottle to which are also added washings from the bags (using distilled water) to bring the total quantity up to a convenient figure

PLATE XIX
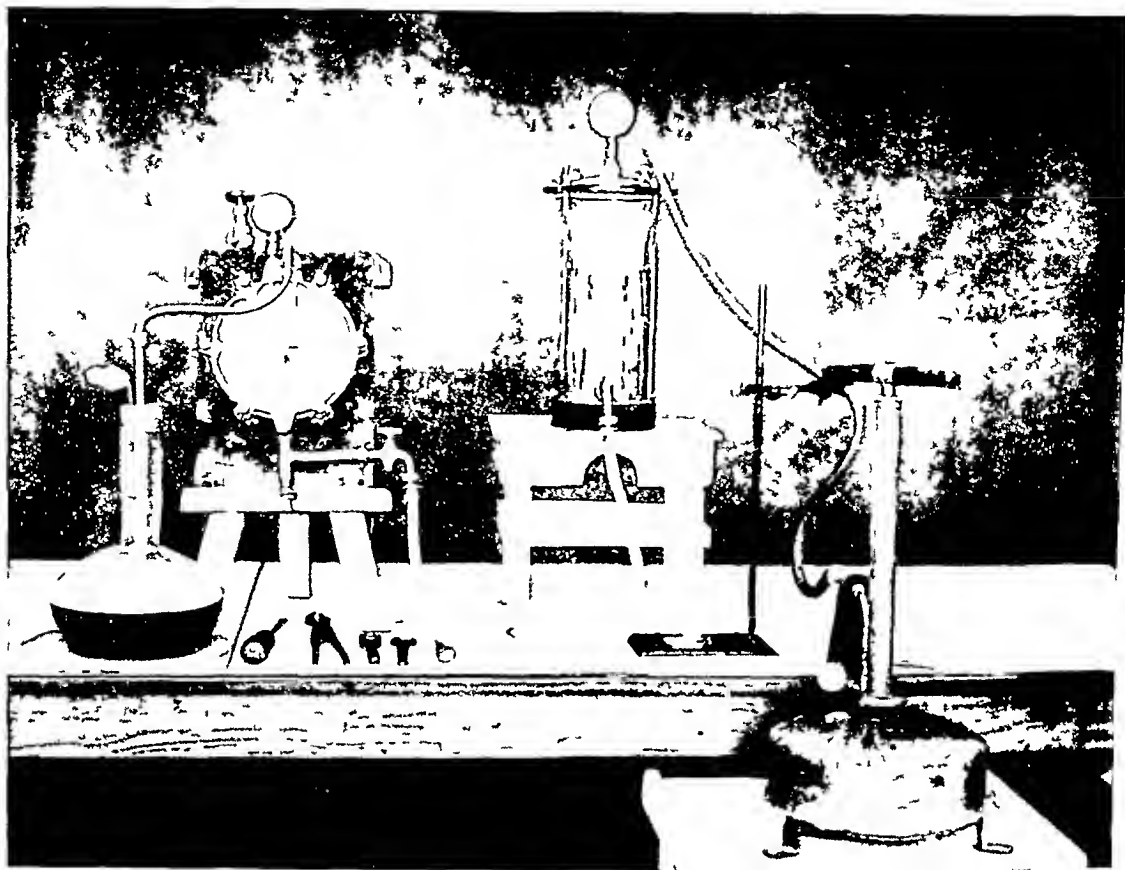
Fig 1   Filtering
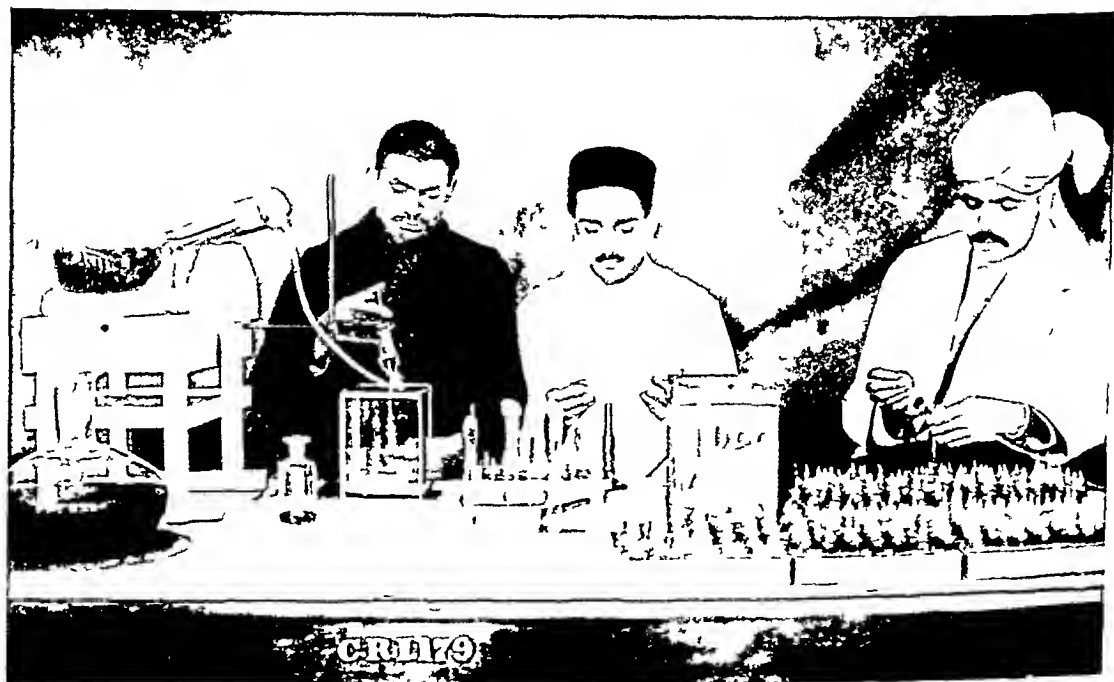
Fig 2   Bottling

The original volume of plasma divided by the volume of the total dialysate gives the degree of concentration

To the dialysate is now added 1 per cent of sodium chloride which at once turns the turbid and viscid fluid into a clear, less viscid and translucent product of slightly greenish hue   The pH is generally found to be between 6 9 and 7 1 and is adjusted to 7 5 or 7 6 with sodium carbonate   A preservative, 0 7 per cent of a mixture of equal parts of ether and trikresol, is next added and the bottle left undisturbed for two weeks   A whitish sediment, consisting mostly of euglobulin (carried over with the pseudoglobulin in the process) and fibres from the chain cloth, collects and clears the product further

A sample is now tested for potency and toxicity   If the titre of protection is satisfactory and the product is not toxic it is passed through a Seitz filter and bottled Plate XIX, figs 1 and 2, show the process of filtration and bottling   The titration of the antivenene and the process of filtration and bottling are described below —

The unconcentrated serum previously issued from Kasauli is prepared by immunizing horses with venom of cobra and Russell's viper   It satisfies the following standard of potency —

(i) One c c mixed with 1 milligram of dried viper venom, dissolved in 1 c c saline, saves a pigeon between 290 and 310 grammes in weight   The mixture is injected intravenously immediately after it is made   The results are available in 5 to 15 minutes   The M L D of the viper venom, when injected intravenously, lies between 0 01 and 0 04 milligram   The serum, thus, protects against 25 to 100 M L D   This test was introduced as a result of Anderson's work (Anderson, 1932)

(ii) One c c mixed with 0 5 milligram of dried cobra venom (dissolved in 1 c c saline) over and above one M L D, and incubated for half an hour at 37°C, protects a pigeon between 290 and 310 grammes in weight   The mixture is injected intramuscularly and the results are read in 24 hours The M L D is determined at the same time thus —

	Venom in milligrams (for M L D)				Serum 1 c c and venom in milligrams (for titre)		
	0 2	0 3	0 4	0 5	0 8	0 9	1 0
Pigeon number	1	2	3	4	5	6	7

Death of pigeon No 3 gives an M L D of 0 4 milligram of venom and survival of pigeon No 6 shows that 0 5 milligram of venom over the M L D has been neutralized by the serum   Similarly, death of pigeon No 4 gives an M L D of 0 5 milligram and the survival of pigeon No 7 shows that 0 5 milligram over the M L D has been neutralized by the serum

The serum thus protects against 2 to 3 M L D only   This test has been in use in Kasauli for many years

The concentrated serum is so diluted as to satisfy the same standard in one fourth of the volume It is issued in doses of 10 c c which replace the former doses of 40 c c of the unconcentrated serum

The toxicity (if any, due to bacterial activity in the later part of the dialysis or any other cause) is tested by giving a rabbit 3 c c of the serum intravenously

The filtration system is closed   The loss due to merely mechanical causes is minimized by washing all the empty containers with 18 per cent solution of ammonium sulphate and storing the washings The filtering discs are also broken up and the protein from them extracted by washing the pulp   The washings are ultimately treated like the diluted plasma to which 18 per cent ammonium sulphate has been added

The bottling system is also closed except at the point of delivery A two way syringe draws up 10 c c of the product, at a time, from the flask and delivers it into the ampoule The needle is protected from dust by a glass case which is sterilized by dipping in cresol and wiped with a sterile towel The adapter and the needle are fitted on the syringe with the aid of a sterile towel and secured in position by a piece of string The ordinary method of filling ampoules in a negative pressure is not applicable as wetting of the neck of the ampoule results in charring on sealing

Any excess of the fluid on the needle is wiped off with a sterile swab dipped in trikresol

Samples for testing sterility are taken from the filling system at the commencement of the process, in the middle and at the end

The loss in protection units in concentration, calculated by estimating the protecting units in the unconcentrated plasma (taking the total quantity of the plasma for serum for the purpose) and in the finished product, was not found to exceed 20 per cent even in the early experimental stages of the work when small quantities were being concentrated In later work at times it appeared as if there was no loss at all This, of course, depends on the gaps in the quantities of the venom and the antivenene and the number of birds used Even a loss of 20 per cent cannot be called excessive

The protein-content of the finished product is below 16 per cent and its viscocity about six times that of normal saline

An example

Batch No Conc A V 6/34   Concentration commenced on 10th May, 1934

Plasma 14,000 c c $\left.\begin{matrix} \\ \\ \end{matrix}\right\}$ $\left\{\begin{matrix} \text{Added ammonium sulphate 8 kilos} \\ \text{Specific gravity 1098} \\ \text{After half an hour specific gravity 1099} \end{matrix}\right.$
Water 28,000 c c
$CaCl_2$ 4 per cent sol 420

Total filtrate measured = 43,420 (including 1,000 c c washing from filters)
Added ammonium sulphate 4 kilos   Specific gravity 1033
Cake 1,166 grammes   Dialysed in 8 bags
Dialysate 2,500   Made up to 2,800   pH 6 8 adjusted to 7 5
Concentration $\frac{14,000}{2,800} = 5$ times

Test of potency —

    A   Against viper venom

        0 8 c c of the unconcentrated serum, from a sample kept, saves a pigeon against 1 milligram

        0 2 c c of concentrated serum also saves

        The protection is at least 4 times

    B   Against cobra venom

        1 0 c c of the unconcentrated serum saves a pigeon against 0 5 milligram of cobra venom over and above the M L D

        0 2 c c of the concentrated serum also saves   The protection is 5 times There does not appear to be any loss of the protecting substance in the process of concentration

Test of toxicity —Three c c of the serum given intravenously to a rabbit
No ill effects observed

Total protein-content 14 91 per cent

Viscosity 5 6 times that of normal saline

Ammonium sulphate present 0 6 per cent

OBSERVATIONS ON THE TECHNIQUE

The antiseptic —0 7 per cent of a mixture of equal parts of trikresol and ether
added to the plasma appears effectually to prevent putrefaction, in spite of dilution
in the later stages of the dialysis when the protecting influence of the salt is removed
A distinct odour of the antiseptic is present in the cake   The writer has only once
attempted, in hot weather, concentration of plasma to which no antiseptic had
been added   The odour of the dialysate was suggestive of putrefaction

The purity of ammonium sulphate —So far salt of three different grades of
purity from three different manufacturers has been used   There does not appear
to be any difference in the product associated with the grade of purity   The salt
should, of course, be free (reasonably) from arsenic and iron

Stored plasma —After several weeks' storage the fibrinogen of the plasma
loses the power of clotting quickly and firmly on the addition of calcium chloride
In dealing with such plasma a heavy white deposit is encountered in the dialysate
during the two weeks' storage   Often a change of colour develops in the stored
plasma   At times the stored plasma thickens uniformly like a soft jelly
No appreciable loss of potency has been found associated with any of these
changes

The colour of the product —As has been mentioned, the colour of the product is
slightly green   On two occasions batches of the product turned brown in the course
of a night   No loss of potency or development of toxicity was associated with the
change in colour

Protein-content, viscosity and potency —No correlations were found between
these three properties of the serum   The increase in potency of the unconcentrated
serum may be associated with a quantitative or a qualitative change in the pseudo-
globulin of the blood   Viscosity depends more on the amount of euglobulin retained
in the product than the total protein-content   In one batch of the product the
viscosity was 12 times that of normal saline (instead of the usual figure 6) while the
protein-content was only 16 6 per cent (instead of below 16 per cent)   The viscosity
decreases as the euglobulin falls out of solution.

Obtaining serum from the plasma —If it be desired to work with serum instead
of with plasma, the latter is diluted with two volumes of tap-water and the usual
amount of calcium chloride calculated for the undiluted plasma added   In six
hours at room temperature a definite but soft clot forms   The clot is squeezed
through muslin   The quantity of fibrin collected on the muslin is negligible and for
all practical purposes the plasma is converted into serum (diluted with two volumes
of water)   The quantity of the antiseptic present is sufficient to prevent putrefac-
tion   When the plasma has been stored for several weeks the time of clotting is
prolonged and may extend to four or five days

A COMPARISON WITH THE SODIUM SULPHATE METHOD

A method of concentrating antivenene with sodium sulphate has been published in this *Journal* (Maitra, Naidu and Ahuja, *loc cit*) The writer finds that the present method has the following advantages —

1 *The temperature.*—The sodium sulphate method needs a constant temperature, the regulation of which, besides being expensive, is inconvenient for work on a large scale

2 *The cost*—Sodium sulphate is more expensive Besides, other reagents, necessary in the process, increase the cost of production still further

3 *The concentration*—The concentration obtained with the sodium sulphate method was found to be on the whole lower than that obtained with the ammonium sulphate method The addition of 3 per cent of sodium carbonate to the cake, in the former method, increases the bulk of the dialysate Besides, it is not conveniently possible to calculate exactly the amount of the sodium carbonate necessary, the weight of the cake depending upon the pressure employed

4 *The colour of the product*—The final product obtained by the ammonium sulphate method is distinctly better in appearance The yellow of the sodium sulphate method is replaced by a very faint greenish tinge

5 *Coloured serum or plasma*—Even highly coloured serum and plasma can be effectually dealt with and the colour practically removed by repeating the process in the second filtration in the ammonium sulphate method The product from such plasma can be used in the process of immunization of horses

6 *Applicability to plasma*—The ammonium sulphate method is applicable to plasma The yield of plasma from the blood is about 20 per cent higher than that of serum It is possible to apply the sodium sulphate method *fractionally* to plasma but the difficulty and inconvenience of regulating the temperature is trebly increased

The only disadvantage of the ammonium sulphate method lies in the prolonged dialysis Between the months of September and June, at Kasauli, however, no evidence of a bacterial decomposition in the product was found The dialysis could be hastened and completed in 24 hours by employing hot running water ($50°C$) for 8 hours, after the cake has liquefied in the cold water There is reason to believe, however, that such heat decreases the potency of the serum

The low potency (and large bulk) of the product reported upon previously in connection with the ammonium sulphate method seems to be entirely due to not employing enough pressure on the cake An excess of 28 per cent ammonium sulphate left in the cake draws in more than its own volume of tap-water in 4 days by dialysis

SUMMARY

1 A brief history of the production of antivenene in India is given The unconcentrated serum was first made in 1901 and the concentrated serum in 1934

2 The technique of concentration is described With a fractional precipitation of the blood proteins with ammonium sulphate the pseudoglobulin is separated and dialysed The dialysate is the concentrated antivenene

3   Observations are made on different points in the technique   0 7 per cent of a mixture of equal parts of trikresol and ether preserves the plasma throughout the process   Any reasonably pure ammonium sulphate serves the purpose   Stored plasma can be used   A change in the colour of the dialysate is immaterial Total protein-content, viscosity and potency of the concentrated serum are not interrelated   Serum can be obtained from the plasma without any appreciable loss

4   A comparison is made with the sodium sulphate method   The ammonium sulphate method is cheaper, easier and more efficient

ACKNOWLEDGMENTS

Acknowledgments are due to Drs J C G Ledingham and G F Petrie of the Lister Institute, London   The writer was given by them full facilities to study the process of concentration of immune sera at their Institute in the summer of 1933

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THE NUTRITIVE VALUE OF INDIAN VEGETABLE FOOD-STUFFS

Part V

THE NUTRITIVE VALUE OF RAGI (*ELEUSINE CORACANA*)

BY

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THE first four parts of this series dealt with the nutritive value of the proteins of ten of the most common Indian pulses   Similar studies have now been extended to some of the Indian millets   This part deals with *Eleusine coracana*, a widely grown millet occupying nearly seven million acres and by far the most important food-crop of Mysore State   It not only has the advantage that it may be left standing on the fields till a convenient time for harvesting, but also that the grain improves by storing and may be preserved for many years without attack by insects or moulds   The millet is known by the following vernacular names Ragi, Rajika, Nachni, Nagli, etc

Narayana and Norris (1928) report the presence of an alcohol-soluble protein in this seed which they have named *Eleusinin*   A more complete analysis of this protein is given in this paper   This protein, however, represents only a small part of the total, most of which has been found to be inextractable by any of the known

protein solvents   The biological value and digestibility of the total proteins of this millet have also been determined by means of feeding experiments

EXPERIMENTAL

Material —A variety called H 22 ragi was obtained from the Hebbal Agricultural Farm, Bangalore   It was ground to flour to pass a 40-mesh sieve and on analysis gave the following percentages —

TABLE I

Ash	Ether extractives	Protein *	Crude fibre	Carbohydrates (by difference)
2 65	1 72	8 37	3 4	83 84

* True protein=8 27

Extraction experiments —Preliminary trials showed that 4 per cent NaCl solution and 70 per cent alcohol were the concentrations best suited to extract the globulins and prolamins respectively   More nitrogen was extracted by alcohol in the hot than in the cold   Distilled water at room temperature (27°C) removed only 7 7 per cent of the total nitrogen

A known quantity of the flour was, therefore, thoroughly extracted several times, first with 4 per cent NaCl and then with boiling 70 per cent alcohol until no more nitrogen was being removed   The residue was treated with 0 2 per cent NaOH to see if any gluten was present   The quantities of nitrogen extracted by each of the solvents are given below —

TABLE II

Material	Solvent	Percentage of total nitrogen extracted
(1) Fresh	Water at room temperature	7 7
(2) Fresh	4 per cent NaCl ,,	19 6
(3) Residue of (2)	70 per cent alcohol (boiling)	20 5
(4) Residue of (3)	0 2 per cent NaOH at room temperature	7 7

Continuing the treatment with alkali for a longer time (3 days) did not materially increase the quantity of nitrogen extracted   Thus, nearly two-fifths of the proteins in this millet is of a refractory nature not being extracted by any of the usual solvents

Probably it is of the nature of the proteins of Alfalfa leaves which were found (Osborne and Leavenworth, 1921) to be soluble only after partial hydrolysis

Preparations of the proteins —The yields of albumin and globulin from the saline extracts were very poor and hence this investigation has been postponed

The method of preparing the prolamin was nearly the same as described by Narayana and Norris (*loc cit*) with slight modifications   The flour was extracted with alcohol at 70°C and the clear extract, after concentrating under reduced pressure was poured into a large volume of 1 per cent NaCl   The prolamin was separated as a brown foamy mass floating on the top   This was washed with distilled water, dissolved in 80 per cent warm alcohol and the solution poured into distilled water with which it formed a white milky emulsion   On gradually adding strong NaCl solution to this, flocculation occurred and the prolamin was precipitated in white flakes   This was centrifuged off, washed thoroughly with water, pressed as dry as possible between filter-papers and extracted in the wet with ether in a Soxhlet apparatus   This served to remove the associated colouring matter and fat, and also to dehydrate the protein   Finally, the protein was dried, powdered and passed through a 100-mesh sieve   The yield was 6 g to 7 g per kilo of flour

Analysis —Thus prepared, the prolamin is a light grey powder giving positive tests for tyrosine, tryptophane, sulphur and phosphorus   The elementary analysis is given below —

<div align="center">

TABLE III

In per cent of air-dry material

</div>

	I	II
Moisture	2 67	2 76
Ash	0 69	0 65
Ash and moisture free—		
Carbon	52 82	53 14
Hydrogen	7 25	7 84
Nitrogen	15 86	15 40
Sulphur	0 79	0 82
Phosphorus	0 16	0 14
Oxygen	23 14	22 66

The nitrogen distribution was carried out by the method already described in Part I (Niyogi *et al*, 1931)   Cystine, tyrosine and tryptophane were determined according to the method of Folin and Marenzi (1929) and the dicarboxylic acids by the methods of Buston and Schryver (1921)   Arginine was estimated both in the basic fraction and directly in the hydrolysate (Plimmer and Rosedale, 1925)

The amide nitrogen results of the van Slyke method may be too high due to de-aminization during continued hydrolysis for long periods (30 hours)   The true amide nitrogen was, therefore, determined by boiling 1 g of protein for 4 hours with 20 per cent HCl (Gortner and Holm, 1917)   The free amino nitrogen was estimated on a one per cent solution of the protein in dilute alkali using the micro van Slyke apparatus and allowing the reaction to proceed for 30 minutes   The free carboxyl groups were determined by titrating a solution of the protein in warm 70 per cent alcohol with $N/14$ alkali   The results are given in Tables IV-A, IV-B, V and VI and compared with other known prolamins —

TABLE IV-A

Nitrogen distribution of Eleusinin *(per cent of total nitrogen)*

Form of nitrogen	I	II
Melanin (insoluble)	1 14	1 02
„ (soluble)	0 39	0 40
Amide .	22 03	21 99
Di amino fraction—		
Arginine	2 94	2 96
Histidine	2 28	2 32
Cystine .	0 46	0 41
Lysine .	0 65	0 52
Mono amino fraction—		
Amino ..	68 96	68 87
Non-amino . .	2 18	2 29

TABLE IV-B

Nitrogen distribution of Eleusinin *as compared with other prolamins*
(per cent of total nitrogen)

Form of nitrogen	Eleusinin of ragi	Prolamin of fenugreek *	Gliadin of wheat †
Melanin (insoluble)	1 08	0 9	0 52
,, (soluble)	0 40	0 4	0 35
Amide	22 0	20 2	24 61
Di amino fraction—			
Arginine	2 95	3 1	6 38
Histidine	2 30	0 7	5 41
Cystine	0 44	1 4	1 68
Lysine	0 59	0 7	0 57
Mono amino fraction—			
Amino	68 92	70 2	53 49
Non amino	2 24	2 3	6 14

* Srinivasarao, Sastry and Narayana (1933)
† Hoffman and Gortner (1925)

TABLE V

Percentages of essential amino acids in Eleusinin *and other prolamins*
(expressed in per cent of total protein)

Amino acid	Eleusinin of ragi	Prolamin of fenugreek	Gliadin of wheat	Method
Lysine	0 5	0 5		Van Slyke
Histidine	1 4	0 4		,,
Arginine	1 45	2 0		,,
Arginine	2 50	2 30		Direct estimation (Plimmer and Rosedale)
Cystine	2 50	3 0	2 19	Folin and Marenzi
Tyrosine	5 3	4 3	3 29	,, ,,
Tryptophane	1 6	2 4	0 83	,, ,,
Dicarboxylic acid calculated as glutamic acid	33 61			Buston and Schryver

TABLE VI

Amide nitrogen, free amino nitrogen and free carboxyl groups

True amido nitrogen (in per cent of total nitrogen)	Eleusinin of ragi	Gliadin of wheat *	Hordein of barloy *	Sativin of oats *	Zein of maize *
Amido nitrogen (4 hours hydrolysis)	22 82	24 24	21 22	21 90	17 25
„ „ (van Slyke)	22 06	24 61	23 38	22 20	18 0C
Difference	0 24	0 37	2 16	0 30	0 81
Free amino nitrogen, in per cent of total nitrogen—					
Free amino nitrogen	1 94	1 86	1 50	1 65	1 19
Half lysine nitrogen	0 29	0 29	1 51	0 60	0 45
Free carboxyl groups—					
N/14 alkali to neutralize free carboxyl groups in 1 g of protein	3 66	2 64	2 52	4 48	2 40
Gramme equivalents of N₁OH required to neutralize 1 g of protein $\times 10^{-6}$	26 13	18 86	18 00	32 00	17 40

* Hoffman and Gortner (*loc cit*)

Metabolic experiments—The methods employed were the same as those described in the previous parts of this series with the exception that low egg-protein diet was substituted for non-protein diets in standardizing the rats   This prevents

the loss of appetite noticed during the non-protein diet period and does not in any
way affect the determination of endogenous and metabolic nitrogens (Mitchell
and Carman, 1926)   As all the millets taken up for investigation contained only
6 to 9 per cent of protein, the level of protein intake was kept at 5 per cent in the
rations instead of 10 per cent as in the previous experiments with pulses   This low
level tends to give high biological values, but the results would, however, serve for
comparison among the millets themselves   Table VII gives the composition of the
rations and the metabolic data are given in Table VIII —

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TABLE VII

Percentage composition of the rations

</div>

	Low egg protein diet	Ragi flour diet
Cane_sugar	10	10
Butter fat	9	11
Cod liver oil	2	2
Agar agar	2 5	0
Salt mixture*	3 0	4
Flour		Enough to contain 5 per cent protein
Dried egg powder	5 0	
Starch	To make up to 100	

* Osborne and Mendel (1920)

The crude fibre contents of both the rations were kept the same by adding
agar-agar to the low egg-protein diet   The egg-powder was prepared by drying
whole contents of fresh eggs at about 70°C   In addition to the rations each rat
received daily 4 drops of cod-liver oil and 50 mg of dried yeast, the nitrogen of
which was not taken into account in calculating the metabolic data

Table VIII

Metabolic data

Rat number	Initial weight (g)	Final weight (g)	Food intake (g)	Nitrogen intake (mg)	Faecal nitrogen (mg)	Urinary nitrogen (mg)	Metabolic nitrogen per g of food (mg)	Endogenous nitrogen per 100 g body-weight (mg)	Food nitrogen in faeces (mg)	Absorbed nitrogen (mg)	Food nitrogen in urine (mg)	Food nitrogen retained (mg)	Digestibility (Per cent)	Biological value (Per cent)
Low egg-protein ration nitrogen = 0 735 per cent														
55	119 0	125 0	10 7	78 66	46 98	32 04	4 390	18 06						
56	112 0	116 5	10 1	74 25	42 53	22 83	4 211	19 97						
57	113 0	119 0	10 8	79 39	45 50	20 77	4 213	17 91						
58	118 0	126 0	11 0	80 87	45 99	22 56	4 181	18 48						
59	107 0	115 0	11 3	83 08	42 29	22 16	3 742	19 96						
60	99 0	104 0	10 3	75 71	51 68	18 99	5 018	18 97						
61	105 5	111 0	9 5	69 84	46 98	20 68	4 945	19 07						
62	121 0	129 5	11 0	80 87	37 10	24 61	3 372	19 64						
Ragi flour ration nitrogen = 0 841 per cent														
55	121 0	120 5	8 83	74 25	56 01	25 59			17 82	56 43	4 96	51 47	76	91
56	115 0	117 0	7 87	66 18	47 23	24 13			12 68	53 50	3 24	50 26	81	04

57	120 5	124 0	9 52	80 04	47 23	24 13	17 07	62 37	9 80	52 57	78	81
58	125 5	128 5	10 32	86 76	62 24	30 99	17 02	69 14	7 99	61 15	80	89
59	116 0	120 0	10 32	86 76	67 47	27 96	23 47	63 20	5 61	57 08	73	91
60	103 0	107 0	8 58	72 14	54 81	22 69	13 21	58 93	3 50	55 34	82	94
61	110 0	109 5	8 33	70 03	55 33	23 86	16 75	49 12	3 18	45 94	76	94
62	128 0	132 0	10 07	84 65	64 49	32 00	21 95	62 70	8 44	55 26	74	87
AVERAGES											77 5	90 5

Low egg protein ration nitrogen=0 735 per cent

55	132 0	132 0	8 13	59 77	34 61	21 76	4 258	16 48
56	124 0	123 0	7 90	58 08	36 10	21 05	4 569	17 04
57	130 0	128 0	8 90	65 43	43 02	21 16	4 834	16 41
58	134 5	133 5	10 63	78 14	47 47	23 82	4 467	17 77
59	127 0	125 0	8 80	64 68	42 13	22 55	4 787	17 89
60	111 0	109 5	7 63	56 10	35 71	19 19	4 679	17 40
61	113 0	111 0	5 90	43 38	25 47	20 77	4 316	18 55
62	137 0	135 0	8 13	59 77	41 30	22 55	5 079	16 59

Conclusions

The presence of phosphorus and the low basic nitrogen content are the essential characteristics of *Eleusinin* of ragi   In both these respects it resembles the prolamin of fenugreek which is the only other prolamin so far reported as containing phosphorus   In its content of cystine, tyrosine and tryptophane it is superior to the gliadin of wheat   The amide nitrogen figure shows that there is not much de-aminization during long and continued hydrolysis   Unlike the globulins of the pulse (*vide* previous parts) there seems to be no relation existing between the free amino nitrogen and the lysine nitrogen of the prolamins

At a 5 per cent level of protein intake, the digestibility and biological value of the total proteins of ragi are 77 5 per cent and 90 5 per cent respectively   At the same level of protein intake the biological values of the total proteins of milk are 93 4 per cent, of rice 86 per cent, and of oats 78 6 per cent (Mitchell, 1924) Thus ragi proteins seem to be superior to those of rice and oats in their biological values   This is quite in accordance with the fact that the local population consider ragi to be superior to rice in nourishing qualities though less palatable

Experiments were also conducted to prepare ragi malt   The diastatic activity of ragi malt was found to be equal to that of barley malt   Since ragi contains a high percentage of starch and is very cheap, it can very well be employed as a basis for the manufacture of farinaceous and invalid foods   Further investigations are yet to be conducted in this field

Summary

Four per cent NaCl and 70 per cent hot alcohol each removes about 20 per cent of the total nitrogen of ragi (*Eleusine coracana*)   The remaining nitrogen is not extracted even by dilute alkalis

The alcohol soluble protein *Eleusinin* of ragi has been isolated and analysed

The biological value and digestibility of the total proteins of this millet have been found to be 90 5 and 77 5 per cent respectively

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THE RELATIONSHIP OF SKIN AND NERVE LEPROSY

BY

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THE classification of leprosy has varied from time to time , but, whatever nomenclature has been used, it has always emphasized the existence of two types, one in which nodulation or thickening of the skin is the most striking feature, and the other in which involvement of the nerves is most prominent It has also been recognized that, while typical cases show either one or other of these features, the one form may merge into the other, the term *mixed* being used to indicate these cases

The Leonard Wood International Leprosy Conference of 1931 applied the terms *cutaneous* (C) and *neural* (N) to the two main types, and sub-classified each type into three grades according to the degree of involvement of the skin or nerves respectively If both skin and nerves are affected a mixed nomenclature is used Thus, if cutaneous (nodular) signs are advanced and neural signs slight, the case is classified as C-3, N-1

While this nomenclature may be more accurate than those formerly used, its interpretation and application are found difficult in certain cases Nor is there much likelihood of improving matters until there is a clearer conception of the factors which determine the relative degree of skin and nerve involvement An attempt is here made to throw some light on this subject

This study can best be made by examining and comparing early lesions of the cutaneous and neural types, material being obtained by biopsy Small elliptical pieces of skin and subcutaneous tissue with nerve branches attached are excised from typical macular lesions Small macules, not more than 5 mm in diameter found in the skin at a short distance from larger similar macules, are the most suitable Their similarity to the larger macules makes their nature recognizable, and as they are small the whole lesion can be excised The main clinical

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distinctions between the neural and cutaneous macule are set down in tabular form as follows :—

Neural macule	Cutaneous macule
Anæsthesia on light touch	Sensation on light touch
Raised and indurated	Flat and soft
Erythema marked.	Erythema slight or absent
Keratosis, anidrosis and depilation marked	Keratosis, anidrosis and depilation slight or absent
Hypopigmentation recognizable, especially if capillaries are emptied by pressure with a watch-glass	Hypopigmentation generally more easily recognized

In cutting sections the material should be arranged so that the nerve lies parallel to the line of section   Alternate sections are stained with a combined bacillary and nuclear stain (as Ziehl and hæmatoxylin), and a combined bacillary and nerve fibre stain

The following table gives the main microscopic distinctions between the two types of macule :—

Neural macule	Cutaneous macule
In the corium dense cellular cords in longitudinal and transverse section with clear cut margins These surround the hair follicles and sweat glands and ducts In the deepest part of the corium they are continuous with the subcutaneous nerves, which have a similar dense cellular structure and are markedly thickened Horizontal branches pass from the main cords, especially at the level of the sub papillary plexus, and these again send branches into the papillæ In some cases the whole superficial part of the corium, including the sub-papillary plexus, becomes one dense cellular mass	There is cellular infiltration round the blood vessels, chiefly in the papillary and sub papillary layers , it may also surround the hair follicles to a certain extent
The cells apparently derived from the endothelial lining of the capillaries, are chiefly of the so called epithelioid type , plasma and mast cells are also present	Cells are similar in type
In the most typical neural macules giant cells of varying size and number are present in the cellular cords and in the superficial part of the corium	Giant cells are absent
Acid fast bacilli may not be found in the skin though careful examination of a number of specially stained sections will generally show a few bacilli	Acid-fast bacilli are seen in varying numbers, chiefly in and around those vessels which show cellular proliferation , the bacilli are found either inside epithelioid cells or in the intercellular spaces

Neural macule	Cutaneous macule
The subcutaneous nerves show marked thickening This is due to cellular proliferation beginning in the core of the nerve or nerve bundle and spreading outwards The nerve fibres are destroyed or displaced outwards by the cellular proliferation, and are found only in the superficial part of the bundle The appearance in longitudinal or slightly oblique section of a nerve bundle stained with Bielchowsky's stain is that of a stalk of asparagus surrounded by a layer of black horse hair	The subcutaneous nerves show practically no thickening There is little or no sign of cellular proliferation
Acid fast bacilli may be difficult to find or they may be found lying in masses between the nerve fibres in the superficial part of the nerve bundle They are generally found in larger numbers here than in the granular cords of the corium In the granular core bacilli are generally not found	Bacilli are more numerous in the subcutaneous nerves than in the corium They lie in large clumps between nerve fibres, especially in the centre of the nerve bundle Many of the bacilli are apparently extracellular
Giant cells of all sizes and often in large numbers are found in the granular core of the nerve bundles Giant cell formation may pass on into caseation and the forming of a cold abscess, one or more of which may appear in the course of a subcutaneous nerve attached to a neural macule Pus evacuated from such an abscess may contain acid fast bacilli singly or in masses, but this occurs only in the more advanced cases	Giant cells, caseation and abscess formation not found Abscess formation is found in leprous nodules in cases of the cutaneous type, especially when ' lepra reaction ' occurs But these abscesses do not occur in the early lesions here described, and they are acute in nature, not of the chronic cold abscess type which follows caseation

It is seen from the above comparison :—

    (a) that there is greater cellular response, in spite of fewer bacilli being present, in the neural than in the cutaneous type of macule ,

    (b) that, especially in the cutaneous type, there is, in proportion to the concentration of bacilli in the corium and nerves, far greater cellular response in the former than in the latter ,

    (c) that the bacillary concentration is greater in the nerves than in the corium, this being most noticeable in the neural type

These three observations combined give a satisfactory basis for explaining the distinction between cutaneous and neural leprosy The neural type shows higher resistance, as is indicated by stronger cellular response accompanied by phagocytosis and destruction of bacilli , hence fewer bacilli are found in sections Cellular response to a given number of bacilli is more easily induced in the corium than in the subcutaneous nerves This is most strikingly seen in the cutaneous macule where a moderate number of bacilli in the corium causes a moderate amount of cellular multiplication and granulation , whereas there is no such response even to the higher degree of infection present in the nerves The same principle is also illustrated in the neural macule with attached subcutaneous nerves, as bacilli are found in larger numbers in the nerves than in the corium The reason for the lower cellular response in the nerves requires investigation It may be due to greater scarcity in the nerves of the cells which take part in the response , or the nerves,

being less exposed than the corium to infections in general, may not have evolved an equally sensitive defence mechanism Apparently, cellular proliferation begins from a small vessel lying in the centre of the nerve bundle and spreads outwards, thus the core of the bundle is always the first part to be affected The spread of infection up the nerve is probably along the lymphatics, as the bacilli are found distributed throughout the thickness of the bundle, though they tend, when cellular response is absent, to be more concentrated in the centre

The nerves thus form a refuge for bacillary infection where it can increase and spread The presence of a high degree of infection in the nerves does not necessarily of itself constitute nerve leprosy This type of the disease is caused only when, in addition to the presence of bacilli in the nerves, there is high enough resistance to induce cellular response to these bacilli, the cellular infiltration causing swelling and tenderness of the nerves, pressure on the axis-cylinder, and consequently neural signs In the cutaneous type, however, hyperinfection (as mentioned below) reduces resistance, and prevents cellular response to the bacilli present in large numbers in the nerves

The relationship between cellular response to the presence of *M lepræ* and the destruction of these organisms as a result of that cellular response is one which should be referred to here In leprosy cellular ingestion of bacilli may apparently be either favourable or unfavourable to the organism There may either be symbiosis with protection and consequent multiplication of the bacilli, or phagocytosis with consequent destruction We express no opinion as to whether one and the same cell is capable under varying circumstances of acting as guest-house and lethal chamber , but there seems no doubt that the stronger the resistance of the patient, and consequently the intenser the cellular response to the presence of bacilli, the more likely is complete phagocytosis to take place This is shown by the relatively small number of bacilli found in the corium of the neural macule in which the cellular response is far greater than in the cutaneous macule It is also shown by the absence of bacilli in the core of the nerve bundle connected with the neural macule, i e , in the part where they have been destroyed through cellular reaction The giant cell may be regarded as the result of a particularly strong cellular reaction to a small bacillary focus , and caseation and abscess formation are probably caused by a similarly urgent reaction to a larger bacillary focus In all the phenomena connected with the neural macule the main determining factor is the sudden passage from low resistance with bacillary multiplication to high resistance with urgent cell response and phagocytosis Where, however, the cellular response is absent or very low, as for instance in the nerves connected with the cutaneous macule, many bacilli may not even be ingested and may thus continue to multiply and form bacillary masses extracellularly

We have shown in previous articles (Muir, 1933, 1934, 1934a) that the principal factors which are responsible for lowered resistance to leprosy are three in number —

(a) *the age factor*—during the first few years of life the resistance to leprosy is lowered even in healthy children ,

(b) *the debility factor*—due to other diseases, dietary error, or other unfavourable circumstances ,

(c) *hyperinfection*—the natural resistance of the body to leprosy is depressed when the concentration of bacilli in the body has increased beyond a certain degree, however strong and healthy the patient may otherwise be

These same three factors, therefore, indirectly influence cellular response to and the phagocytosis of *M lepræ* Inversely mild (subliminal) infection with leprosy tends to raise the resistance of the patient to leprosy and intensify cellular response to and phagocytosis of *M lepræ*

The manner in which the principles formulated above govern the formation of various types of lesions may best be illustrated by describing a few cases —

As the first case we take the example of a naturally resistant patient who has a single focus of infection in the skin of the back of the hand showing no clinical signs For three months he is debilitated by an attack of malaria, after which he recovers During the period of debilitation the resistance of the patient is lowered to a certain extent The cellular response is sufficient to prevent the spread of infection along the vascular plexuses of the corium , but, as the cellular reaction to infection in the subcutaneous nerves is less than in the skin, the bacilli are able to spread up the nerve branches, the distance of spread and concentration of bacilli varying with the duration of lowered resistance In this way a more or less pure form of nerve leprosy is formed During the period of depressed resistance no recognizable signs or symptoms may be present , but, when the patient has recovered from the malarial attack and has regained his former good health and high resistance, there begins to take place a cellular response to the bacilli which have meanwhile multiplied and accumulated in the nerves and their branches It is then that sensory and trophic changes take place in the parts supplied by the affected nerves , also, if the concentration of bacilli anywhere in the nerves be sufficient, there may be noticeable thickening and tenderness and even occasionally abscess formation

In a second case the degree of temporary debility and lowered resistance is greater than in the first The spread of infection is not checked in the cutaneous capillaries, but spreads radially through the skin from the original focus to an extent relative to the degree and duration of the depressing factor At the same time infection spreads up the cutaneous nerves Here again clinical signs will be seen not during but after the period of debility The appearance of the lesion in the skin will vary according to circumstances If resistance has been depressed only to a mild degree, a certain amount of cellular response and destruction of bacilli will have taken place at the centre, while the active' infection has continued to spread radially When, therefore, resistance is restored there will be more bacilli present, and therefore, more cellular reaction, at the margin, which will become thickened and indurated, the centre remaining comparatively flat and soft If, however, resistance has been still more depressed, the central phagocytosis will not have taken place and the bacilli will be more equally distributed through the whole affected area Thus, on restoration of reacting power, the whole macule will become equally raised and indurated In this type of case also it is common to find considerable thickening of nerves with trophic and sensory changes

In a third type of case the period of debility and depressed resistance has lasted for a considerably longer period, and multiple lesions have been formed When the depressing factor, say some intercurrent disease, has been removed the bacilli have

already multiplied in the body to such an extent that the threshold has been passed between subliminal infection and hyperinfection   Recovery from debility is accompanied by increased cellular response , but, considering the bacillary concentration, which has become far higher than in the first two cases mentioned, the cellular reaction is comparatively of a much milder type   Ingestion of bacilli takes place without true phagocytosis, and destruction of the bacilli is unable to keep pace with their multiplication   Such a lesion may, to begin with, show little thickening or induration, though these tend to increase as the infection increases and affects the whole depth of the skin   In this type nerve thickening and trophic and sensory signs are, if present, much less noticeable , the bacilli, though present in large numbers in the nerves, fail to excite much cellular response

A fourth type of lesion is caused by a still more prolonged and/or severe depression of resistance, in which any or all of the three depressing factors (age, debility, hyperinfection) may take varying shares of responsibility   Infection having spread by metastasis sets up multiple foci in the skin   From these foci radial spread takes place till the lesions coalesce resulting in entire, or almost entire, infiltration of the skin   The infection may be chiefly confined to the sub-papillary plexus, or the subcutaneous plexus may also be involved   In such cases, though there is mild cellular response to the large number of bacilli present, the hyperinfection factor (aided or not by the other two factors) tends to prevent effective phagocytosis and destruction of the bacilli   If there is a greater concentration of bacilli at any point, then local cellular multiplication takes place there causing the familiar leprous nodule   In many cases, however, nodulation may be absent, and occasionally the patient may present clinically an appearance which even to those with considerable clinical experience would scarcely raise the suspicion of leprosy   The condition of the nerves is similar to that in the third type

The fifth type appears as a continuation or final stage, especially of the last two types   Advanced leprosy has a tendency towards self-healing   As the bacilli become eliminated from the skin and internal organs the hyperinfection factor diminishes until a point is reached at which the masses of bacilli sheltering in the nerves are able to excite marked cellular response   In consequence of this, tenderness and thickening of the nerves with trophic and sensory disturbances become the most marked features in the clinical picture   In this type we have trophic ulcers, wasting of the small muscles of the face, hands and feet, and deformity and destruction of the fingers and toes

To complete the series it is necessary to describe a sixth type, viz , that found in young children and especially familiar to those in medical charge of homes for the children of leprous parents   Clinically there are hypopigmented areas of the sk⁻ with slight roughness and keratosis   On section the skin shows cellular infiltration chiefly round the sub-papillary vascular plexus   Bacilli are often scanty and difficult to find   History of close contr   n infectious case may be necessary to confirm the diaᵣ   f leprosy   -   hanges may be difficult to elicit
The infection has s`   ly due to t   or lowering the natural resistance
of the patient, and   ·⸗⸗in prop(se cases recover if removed early
enough from the so   tion, maᵤ   ough h⁻⁻⁻rinfection or debility
or both these factc   , pass oı   ⁻re d   forms of advanced
cutaneous leprosy

The leprolin test —Mitsuda's leprolin test (Chiyuto, 1932 , Hayashi, 1933 , Muir, 1933) throws much light on the nature of leprous lesions and tends to confirm some of the above-mentioned interpretations of the phenomena of leprous lesions Leprolin consists of an autoclaved suspension of ground-up leprous nodule containing large numbers of Hansen's bacilli If 0 2 c c of leprolin is injected intradermally into the healthy skin of a leprous subject, a local lesion is produced having clinical and histological resemblances to his already existing lesions If the patient's macules are hard and indurated and of the highly resistant type, showing compact cellular cords, numerous giant cells and few or no bacilli, then the leprolin nodule, sectioned 3 or 4 weeks after the time of inoculation, will show similar dense cellular infiltration and numerous giant cells If the patient's lesions are of low resistant type, then no leprolin nodule appears, and sections of the skin at the point of inoculation show little or no cellular response The leprolin test is positive in most healthy non-leprous adults , it is diminished or negative in young children, in debilitated subjects and in those with a leprous hyperinfection , it is increased in leprous subjects who are otherwise healthy, and in whom infection has only been slight We have not seen bacillary invasion of local nerve branches as the result of the injection of leprolin In resistant cases the dead bacilli of the leprolin are rapidly phagocytosed , in non-resistant cases the bacilli are apparently carried away by the lymphatics of the skin, though whether any of them enter the attached nerve branches it is difficult to say

The value of the leprolin test is considerably enhanced by using as a control an autoclaved suspension of Stefansky's bacillus (*M lepræ muris*), obtained by grinding up the liver, spleen and omentum of rats previously inoculated with this organism This suspension is known as Stefansky's leprolin in contrast to Hansen's leprolin prepared with *M lepræ* It is standardized so that 0 2 c c , injected intradermally into healthy non-leprous adults, produces in 3 weeks' time a nodule similar in size to that produced by the same dose of Hansen's leprolin Like Hansen's leprolin it gives only slight or negative results in young children and debilitated adults , but it differs in giving positive results in non-debilitated adult lepers of the cutaneous (C) or non-resistant type

The leprolin test tends, therefore, to confirm the hypothesis that the nature of leprous lesions is chiefly determined by the degree of resistance to the disease and by the degree, duration and frequency of lowered resistance caused by one or other, or a combination, of the three factors tender age, debility from whatever cause, and hyperinfection The greater the resistance the more conspicuous are the neural lesions , and the less the resistance the less prominent are clinical signs and symptoms affecting the nerves

CONCLUSIONS AND DISCUSSION

1   Leprosy infection tends to spread along the line of the vascular plexuses of the skin (sub-papillary and subcutaneous) and up the cutaneous nerve branches from the skin

2   The multiplication of *M lepræ* and the spread of infection through the skin, nerves and other organs of the body is —

(*a*) in inverse proportion to the degree of natural and acquired resistance of the patient to infection, and to the degree of endoth

cellular response evoked locally by the presence of *M leprœ* in the tissues, and

 (b) in direct proportion to the degree, duration and frequency of the depression of this resistance

 3 The degree of local cellular response evoked by the presence of *M leprœ* in the tissues is in direct proportion to the local concentration of these organisms, and to the natural and acquired resistance of the patient, and in inverse proportion to the depressing factors (age, debility and hyperinfection) Cellular response is a function of local bacillary concentration and resistance, which may be symbolically expressed as follows CR = f (LBC×R), where CR = cellular response, LBC= local bacillary concentration, and R = resistance

 It has been suggested that neural leprosy may possibly be the result of a neurotropic strain of *M leprœ*, but there is little or no evidence to support this view

 4 Either higher bacillary concentration or a greater degree of resistance is necessary to cause the same degree of cellular response in the nerves as in the dermis The cause of this is unknown, but it may possibly be due to comparative scarcity in the nerves of the endothelial cells which take part in this response, or it may be that endothelial cells contained in the nerve bundles are less sensitized to infections in general and are, therefore, slower in responding to the presence of *M leprœ*

 5 In young children and in debilitated subjects the pathway between the milder and grosser infection is open, and the one tends to pass, often rapidly and imperceptibly, into the other But in the case of subjects who have passed the non-resistant early age period, and in whom debilitating factors are not present, there is a marked hiatus between the mild type in which subliminal infections have increased the resistance, and the grossly infected type in which hyperinfection has lowered the resistance The majority of leprosy cases can, therefore, be classified as *resistant* or as *non-resistant* cases, the latter being sub-classified as non-resistant through age, debility or hyperinfection, or through combinations of these factors

 6 There is strong evidence to show that, as a rule, leprosy is not progressive in resistant cases, provided that the general health is not depressed severely or for a prolonged period This is borne out by careful investigation into the history of infected families (Muir and Chatterji, 1934) Therefore, in the treatment of such cases, the chief emphasis should be laid upon the maintenance of good health and the avoidance of debilitating factors

 7 Although bacteriological examination of skin lesions in resistant cases may fail to show *M leprœ* or show only very few, examination of the nerves supplying the macules in these cases will frequently show a larger number of these organisms, a higher degree of resistance being necessary to destroy the bacilli in the nerves than in the skin

 8 The fact that subliminal infection with *M leprœ* increases immunity to leprosy suggests that injections of suspensions of *M leprœ* in the form of diluted Hansen's leprolin should still further increase the immunity of resistant cases of leprosy Also the immunizing effect of these organisms should be stronger if they are injected intradermally into and round the lesions which are already showing a cellular response to *M leprœ*

It has been noted that in the first two types of cases mentioned above the degree of resistance was sufficient or almost sufficient to cause entire destruction of *M lepi æ* in the skin but not in the connected nerve branches, these latter tending to act as reservoirs of infection   If, however, by intradermal injections and possibly by perineural subcutaneous injections of the leprolin the local and general resistance can be enhanced, phagocytosis and destruction of the intraneural bacilli may be induced or amplified

With this object in view we have treated patients with lesions of the second type described above with intradermal infiltration of dilute Hansen's leprolin into macules, and an area half an inch in width beyond their apparent margin   The degree of dilution of leprolin is in proportion to the reaction obtained with a preliminary leprolin test   A 1 in 10 to 1 in 20 dilution of the stock leprolin is generally used   At the same time or at a later sitting the subcutaneous tissue round palpably thickened nerves is infiltrated, care being taken to avoid injection into blood vessels, by pulling on the piston of the syringe before pressing it home   It is still too soon to report on this form of treatment, but the results obtained so far are promising Success is indicated by a stronger reaction to the leprolin test and disappearance of active signs in macules and connected nerves   In several cases thus treated the lesions have completely resolved after one or more such infiltrations, thickened and tender nerves clearing up at the same time, though more slowly   One great advantage of this form of treatment, especially to those living at a distance from the treatment centre, is that the patient does not necessarily require to pay many or frequent visits , appearance at intervals of one or two months may be sufficient in some cases

9   Obviously, leprolin treatment such as that suggested here could not be expected to be useful in the third, fourth and sixth types of lesions mentioned above, that is to say in patients in whom resistance has been lowered by hyperinfection In such cases injection of leprolin would only still further depress resistance   We have, however, obtained encouraging results in the third type, when the infection was strictly limited, by intradermal infiltration of the lesions with diluted Stefansky's leprolin   In these patients the reaction to Hansen's leprolin test was negative, but positive to Stefansky's   Infiltration of lesions with the latter suspension (diluted 1 in 5 to 1 in 20 in inverse proportion to the degree of reaction to the test) produced local cellular reaction and phagocytosis of both the injected leprolin organisms and the formerly present Hansen's bacilli , whether or not the injection of Stefansky's leprolin in such cases produces any degree of group immunity is a matter for investigation

10   Another use of the leprolin test is in estimating the resistance of those who have been in contact with an infectious case, especially in the case of children In those who react satisfactorily and are, therefore, resistant cases further resistance may be induced by occasional (every 2 or 3 months) injections of leprolin

11   One of the most searching criteria which can be used as a guide before discharging arrested cases is the leprolin test   When a former C-2 or C-3 case becomes bacteriologically negative and remains so for two years the signs are encouraging   The patient should, however, be kept under observation unless or until the reaction to Hansen's leprolin becomes at least moderately strong   This

test is also useful in determining the length of treatment required in cases of the nerve type

SUMMARY

The distinction between neural and cutaneous leprosy is explained by the aid of histological findings and the leprolin test   The main factor determining the one type from the other is shown to be the degree of resistance   Another important factor is the relatively low resistance and cellular response to lepra bacilli in the subcutaneous nerves as compared with the skin   The importance is dwelt on of the distinction between resistant and non-resistant types in the diagnosis, prognosis, prevention and treatment of leprosy

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CUTANEOUS LEISHMANIASIS AS A NATURAL INFECTION OF A DOG IN INDIA

BY

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THE occurrence of natural infections with *Leishmania* does not appear to be very uncommon among the dogs of countries on the Mediterranean littoral and in some parts of Persia, Iraq, Transcaucasia and Transcaspia   In these countries both visceral and cutaneous canine leishmaniasis have been recorded either as separate diseases, or in combination

Although some thousands of dogs were examined by Donovan, Patton and Mackie, in areas of India where human visceral leishmaniasis is endemic, in no case was infection with *Leishmania* found   The same absence of visceral *Leishmania* infection in dogs was a feature of the epidemiology of kala-azar in Assam, even in the midst of the epidemic centres of the disease during the last outbreak   One of us (H E S) working in Assam in 1923 during the height of the kala-azar epidemic, made an examination of dogs with a special view to discovering any natural visceral *Leishmania* infections among them

The examinations were considered more likely to detect canine infections in an area where human kala-azar was very prevalent, if they were confined to animals showing marked signs of ill-health, as exhibited by emaciation, loss of hair, etc , rather than extended to a larger number of dogs taken at random   In this way it was hoped the more readily to detect possible infections, for two reasons —

(a) Dogs showing signs of ill-health were more likely to be the infected ones, and

(b) The limitation of numbers enabled a very thorough examination of each dog to be made

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Such a series of dogs had probably a greater value than many times their number of unselected animals

A total of 44 dogs was examined   Each dog in the series was examined by autopsy   Direct smears and cultures on N N N medium were made from the liver, spleen and bone-marrow in 43 and smears only in one   All the findings were negative as regard *Leishmania* infection*   Only two cases of natural infection with this parasite have been recorded in dogs in India   Both these were cutaneous The recognition of a third case appears worthy of note

Avari and Mackie (1924) examined a superficial ulcer near the base of one of the ears of a pariah dog in Bombay, and found *Leishmania* present   When the animal was seen again about a week later, the sore had almost healed   These authors mention another case of this infection occurring at the same time in Bombay   The latter has been described in greater detail by Row (1925)

Row (*loc cit*) examined a 'quasi-bulldog' which had extensive ulcers on the lips, ears, nose and the inner canthus of the left eye   In addition there were a number of non-ulcerated lesions on the inner sides of the ears, looking like shining buttons of the size of a large pea   The animal had arrived in Bombay shortly before from Landi Kotal, in the North-West Frontier Province of India   *Leishmania* were found in these lesions, and it was suggested that the parasites differed in some particulars from those seen in human cases of this disease

Row (*loc cit*) was unsuccessful in obtaining cultures of the parasites, and intra-peritoneal injections of material obtained from the sores failed to infect two mice which were observed from 2 to 6 months afterwards   Two pariah dogs were injected at the same time subcutaneously in the ear   A nodule appeared at the point of injection after about 6 weeks in both dogs   This ulcerated and showed O-bodies These experimental lesions healed spontaneously after about $4\frac{1}{2}$ months from the original inoculation

The pariah dog of the earlier record was captured in the vicinity of the Veterinary Hospital where Row's case was undergoing treatment at the time   As both cutaneous and visceral leishmaniasis appear to be rarely, if ever, contracted in Bombay, Row (*loc cit*) suggests that the infection reported by Avari and Mackie (*loc cit*) may have been contracted from his case

DETAILS OF THE PRESENT CASE OF CANINE LEISHMANIASIS

The animal was a white bull-terrier, male, aged 3 years   He was born and lived most of his life at Kasauli in the outer Himalayas (6,000 feet above sea-level) In December 1933, he was taken to Karnal, Punjab, where he remained until the middle of May 1934, when he returned to Kasauli

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* While this paper was in the press, we have received a publication by Cammopetros (1934) which states, 'La leishmaniose naturelle cutanee du chien est inconnue jusqu'a present en Mediterranee, elle est decrite dans quelques regions d Asie, en particulier a Teheran où elle est tres répandue ainsi qu'au Turkestan et a Delhi (Indes)'   We have been unable to trace the origin of the statement that canine cutaneous leishmaniasis is common at Delhi   The only reference we can find to such a possibility is that of James (1905) who examined sores on dogs in that city and states, ' the generally accepted opinion that true Delhi sores occur on dogs and other domestic animals has yet to be confirmed '

On 16th July, a number of small, pale pinkish, raised nodules were observed on the animal's nose   The lesions were situated on the almost hairless skin just outside the corrugated sensory area of the nose   On the upper surface of the nose were three nodules about 3 mm in diameter, and three others about half this size   On the left side of the nose, just below and behind the slit of the left nostril, was a group of four confluent nodules, each about 3 mm across   Half-way along the upper lip on either side, on the skin near the edge of the mucous membrane, was a small nodule about the size of those last mentioned

These pale nodules were slightly raised and their surface seemed smoother than that of the surrounding skin   Each nodule was slightly umbilicated and, when examined with a lens, this appeared to be caused by some commencing destruction of the skin, although it was difficult to be certain of any actual breach in the surface   One of the nodules near the nostril had a distinct central ulcer about 1 mm across   The lesions were of such a slight nature that, but for their contrast against the black skin around them, they might easily have been overlooked   A careful search was made but no other nodules could be found anywhere on the body

Smears were made of scrapings from some of these lesions, and, when these were stained with Giemsa's stain, typical Leishman-Donovan bodies were found The parasite was also successfully cultured on N N N medium

DISCUSSION

The question arises as to where the animal acquired the infection   The dog had been under close observation since birth, and the only two places in which he had ever spent a night, are Kasauli in the Simla Hills, and on the plains at the Ross Field Experimental Station for Malaria at Karnal, Punjab

When *L donovani* is injected into dogs, the lesions produced are sometimes cutaneous in character   It, therefore, appears necessary to consider the possibility of infection with *L donovani* in addition to *L tropica*

Leishmaniasis of any kind is unknown in Kasauli as an infection acquired locally   Kala-azar has, however, been reported on a number of occasions among the children in a school on the neighbouring hill-top (Sanawar) about 2 miles away The two places are separated by a wide and deep valley, and while *P argentipes*, the probable vector of kala-azar, has been found in Sanawar, it has never been recorded in Kasauli   Both stations, however, have large numbers of *P chinensis* and *P major* during the summer   Of these insects the former is suspected to be the vector of kala-azar in North China, and the latter of the same disease in the Mediterranean area

At Karnal, oriental sore is not uncommon, but kala-azar has never been recorded In the Ross Field Experimental Station for Malaria sandflies are numerous, and *P papatasii*, *P sergenti* and *P argentipes* have all been collected   The first two species of insect are strongly suspected of being the carriers of human cutaneous leishmaniasis in southern Europe, North Africa and western Asia

The absence of lesions from the more hairy parts of the body negatived the view that the infection could have been carried by fleas, or similar more personal insects   The fact that they were localized to an exposed area where hair was almost

entirely absent* suggests carriage by a flying insect with a short proboscis The experimental evidence in connection with human forms of leishmaniasis suggests very strongly that flies of the genus *Phlebotomus* carry the infection in nature

It does not seem probable that the infection was acquired in Kasauli because (*a*) no local cases of leishmaniasis, either cutaneous or visceral, have been recorded, (*b*) none of the suspected vectors of cutaneous leishmaniasis (*P papatasii* and *P sergenti*) occur in Kasauli, and (*c*) another dog of the same species, living under the same conditions and in the same house in Kasauli has shown no lesions

At Karnal on the other hand, (*a*) local cases of cutaneous leishmaniasis are not uncommon, (*b*) *P papatasii* and *P sergenti* occur in close proximity to the sleeping place of the animal, and (*c*) another dog, which was living under the same conditions but did not have a prolonged stay on the plains during the sandfly season, has shown no signs of the disease

From the evidence available, it appears almost certain that the infection was acquired at Karnal, Punjab

SUMMARY

A case of cutaneous leishmaniasis in a dog is reported The infection was probably acquired at Karnal, Punjab.

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* This animal was observed while curled up in sleep, and practically the only hairless portion of the body exposed at such times was its nose On several occasions gorged sandflies have been observed resting on the wall above the sleeping animal

THE USE OF BACTERIOPHAGE AGAINST CHOLERA IN NORTH ARCOT DISTRICT, MADRAS PRESIDENCY, IN 1933

BY

K C KUTTY ETTAN RAJA, L R C P & S, D P H, D T M & H (Camb)

(From the Public Health Department, Madras)

PROPHYLACTIC ADMINISTRATION OF BACTERIOPHAGE

1   THE first clear indication in Madras Presidency that bacteriophage might be useful against cholera was at the Kistna Pushkaram festival in August 1933 lasting two to three weeks   In the case of this festival, which is celebrated at twelve-year intervals, previous records show that outbreaks of cholera used to be associated with its observance   On these occasions, any epidemic of cholera prevalent in the surrounding area used to invade the festival centres and the celebration of the festival led to exacerbation of the epidemic   On the present occasion there was no cholera in any of the festival centres   The neighbouring districts of East and West Godavari started cholera in July 1933 and the epidemic continued through August, September and October   Even in Kistna and Guntur districts, where the festival took place, there were infected villages   From the infected villages of all these districts hundreds of pilgrims came to Bezwada, the most important centre, and the other places of worship along the course of the Kistna river   However, there was not even a single case of cholera though the festival lasted two to three weeks   The suggestion, therefore, was that bacteriophage must have contributed to the absence of cholera, because the usual sanitary measures were the same on this as on previous occasions

2   As soon as the Kistna Pushkaram was over, experiments with bacteriophage were instituted in the Godavari districts   Certain villages were taken as controls and others for distribution of 'phage   In both the groups the usual measures against cholera, including anti-cholera inoculation, were carried out, and in one group bacteriophage by oral administration (as the water-supply was from canals and tanks) was also carried out   Dr Ethirajulu Naidu, Assistant Director of Public Health, inspected these villages and he was of opinion that the figures supplied from these villages were not reliable   Hence, they have not been included in this report

3   In November further experiments were started in North Arcot district The experiments were conducted on the same lines as in the Godavari districts except that, in villages with wells for their water-supply, the administration of prophylactic bacteriophage was carried out by 'phaging the wells   Chlorination preceded the addition of bacteriophage   Every cholera-infected village had chlorination of wells, whether it was a 'phaged or a control village   In the case of the 'phaged villages there was no further chlorination after the first one, while in the case of the control villages it continued.   As regards the 'phaged villages, after the lapse of about 36 to 48 hours, the water was tested for the presence of free chlorine with starch and potassium iodide and, in the absence of chlorine, bacteriophage was added at the rate of one ampoule of 20 c c for every 1,800 gallons of water in the well   This was the instruction issued by the Public Health Department after consulting Dr I N Asheshov   A complete census of each village ('phaged and control) was taken just prior to the starting of the experiment and the number of houses supplied by each well was noted   On the basis of the population of these houses, the water drawn off from each well was calculated at the rate of 5 gallons per head, which is indeed a very liberal allowance for the average villager in the district   On the second and third days after the first 'phaging, bacteriophage was added to the well in quantity corresponding to the water likely to have been drawn off (which would have been replaced by the springs) and every fourth day the full amount of 'phage necessary for the contents of the well was put in

4   As regards oral administration a fourth of the ampoule (5 c c) of bacteriophage' was added to about three gallons of water in a vessel and each individual was made to take a mouthful of the water   The sanitary inspector had to carry out the administration early morning and late in the evening in order to ensure that the maximum number of persons possible received bacteriophage   As far as possible those who were missed in the morning were traced out and given 'phage in the evening   The register of daily administration of 'phage was marked on the spot

5   The bacteriophage used throughout the experiments was a mixture of cholera 'phage types A, B, C, D and E and was manufactured at the King Institute, Guindy, from a ' passaged ' seed, that is, one which had been carried through cholera patients and was found to yield a potent 'phage on cultivation   This ' passaged ' seed was obtained from Dr Asheshov who was also using the same seed for his manufacture at the time.   The same strain of cholera—recently isolated and smooth—was used throughout for the manufacture of the 'phage

6   It is true that the dose given by oral administration was definitely higher than that given through the wells   In this study no attempt was made to separate the figures for the two methods of administration   In the first place, the figures are so small that separation will further minimize their value for statistical analysis   Secondly, if the dosage given through the wells, in accordance with Dr Asheshov's suggestion, should suffice as the minimum necessary for prophylaxis, larger doses by the method of oral administration should have the result of making the effect of bacteriophage more significant, if it had any prophylactic value   In this study there are 23 attacks among persons receiving prophylactic 'phage and of these only 8 attacks relate to administration through the wells   In their case the number of consecutive days of administration of bacteriophage just prior to the attack ranged from 2 to 24 days

7   In every village where bacteriophage was being used and in every control village there was a separate sanitary inspector posted for duty in order to ensure that, as far as possible, the same amount of skilled observation was rendered available in both the groups of villages, as absence of uniformity would have a disturbing influence in determining the course of the epidemic in the experimental villages   Dr K Sankaran, the District Health Officer, was in immediate charge of the experiments and, in order to enable him to devote himself to the supervision of the work of the sanitary inspectors, he was relieved of his routine duties by the posting of an Assistant District Health Officer   Dr B Ethirajulu Naidu, Assistant Director of Public Health (Epidemics), controlled the experiments and by periodical inspections, ensured that they were conducted properly   Further, between the 5th and 10th January 1934, Dr Naidu and the author inspected these villages together and by field inquiries attempted to assess the value of the data collected   The figures are few but we are satisfied that they are reliable   The reason for the smallness of the figures is that, by the time the village headmen reported cholera and the health staff arrived on the scene and started operations, the epidemic had died out in the case of several villages and they had to be left out of this study

8   At the suggestion of Lieut -Colonel H H King, I M S, Director, King Institute, Guindy, the attacks and deaths were divided into four classes   Class I relates to the figures before the arrival of the health staff on the scene and class II to the period of partial anti-cholera measures   What is meant is that, during this period, the sanitary inspector was not in residence at the village and that he carried out, whenever he visited the village, chlorination of wells, inoculation of contacts and disinfection of the infective materials in the houses where there were attacks during his visit   Class III gives the attacks and deaths after an inspector was placed in the village to be in permanent residence and this represented the period of intensive anti-cholera measures   'Phaging operations relate to this period   Class IV gives the figures for a period of 14 days after intensive operations were started   Thus class III is inclusive of class IV

9   In assessing the value of bacteriophage we are concerned only with the figures of classes III and IV as the administration started with the permanent residence of an inspector in the village and the institution of intensive operations   For the purpose of securing figures of attacks and deaths for an equal length of period of exposure to infection in the case of both the 'phaged and un'phaged villages, Lieut -Colonel King suggested that a period of 14 days might be taken from the date of starting intensive anti-cholera measures   The number 14 was arrived at by the addition of ten days (an arbitrary figure adopted as the period of persistence of infection in the community after the last attack) to the shortest period of prevalence of the epidemic in these villages (4 days) from the date of starting intensive measures   This date differed from village to village, that is, the institution of such measures took place at varying points in the curves of the epidemic in the different villages   In this sense we are combining dissimilar experiences, because cholera tends to rise rapidly to its maximum and then declines both as regards its incidence and the rate of mortality   However, the division into the four classes mentioned above is perhaps the best means of securing comparable figures

10   In each of the above classes the attacks and deaths were divided *into four groups in the case of the 'phage villages and into two groups in the case of the control*

Is the difference in these rates significant, though the 'phaged population shows a smaller rate ?

<div align="center">TABLE I</div>

<div align="center">*Prophylactic treatment with bacteriophage*</div>

	Not attacked	Attacked	TOTALS
Not 'phaged	1,831	28	1,859
'Phaged	1,445	19	1,464
TOTALS	3,276	47	3,323

$$\chi^2 = \frac{3,323 \ (1,831 \times 19 - 1,445 \times 28)^2}{1,859 \times 1,464 \times 47 \times 3,276} = 0\ 255$$

$$P = 0\ 614$$

The value of P shows that the difference in the rates may be due solely to chance

(2) Taking the 'phaged villages alone, a comparison may be made between the rates of attack of the populations of groups II and IV of Statement I ('phaged only' with 'given neither 'phage nor vaccine') It is true that, as bacteriophage is said to spread rapidly through the members of a community, even when its administration is confined to a portion of the population, group IV must have received small doses of bacteriophage, though not in the amounts received by the population of group II However, if bacteriophage is definitely useful against cholera, the difference in the rates should be significant as group I alone received effective doses of 'phage

<div align="center">*'Phaged villages*</div>

Population at risk ('phaged only)	Attacks	Rate per mille of population	Population at risk (given neither 'phage nor vaccine)	Attacks	Rate per mille of population
1,464	19	13 00	318	7	22 01

Here again, the 'phaged population shows a smaller rate   Is the difference in the rates due to chance ?

TABLE II

Prophylactic treatment with bacteriophage

	Not attacked	Attacked	TOTALS
Not 'phaged	311	7	318
'Phaged	1,445	19	1,464
TOTALS	1,756	26	1,782

$$\chi^2 = 1\ 483 \qquad P = 0\ 223$$

This value of P shows that the difference in the rates may be due solely to chance

 (3) A comparison of the attack rates between the combined populations of groups II and IV of the 'phaged villages and group II of the non-'phaged villages thus excluding the inoculated populations in both 'phaged and non-'phaged villages   We may use the figures of class III in both cases so that we are comparing the total experience in the 'phaged and non-'phaged villages during the period of intensive anti-cholera measures

'Phaged villages

Population at risk (groups II and IV combined)	Attacks	Rate per mille of population
1,782	29	16 27

Non-'phaged villages

Population at risk (not inoculated)	Attacks	Rate per mille of population
1,855	33	17 8

TABLE III

Prophylactic treatment with bacteriophage

	Not attacked	Attacked	TOTALS
Not 'phaged	1,822	33	1,855
'Phaged	1 753	29	1,782
TOTALS	3 575	62	3 637

$$\chi^2 = 0\ 125 \qquad P = 0\ 724$$

This value of P shows that the difference in the rates is not significant and may be due to chance

If we take the figures for class IV instead of class III, as we did above, the result is the same Table IV gives figures for class IV

TABLE IV

Prophylactic treatment with bacteriophage

	Not attacked	Attacked	TOTALS
Not 'phaged	1,831	28	1,859
'Phaged	1,756	26	1,782
TOTALS	3,587	54	3,641

$$X^2 = 0.014 \qquad P = 0.907$$

Here too, the difference in the rates is not significant From all these tests we have to conclude that, with the available data, bacteriophage when it is administered as a prophylactic agent in the manner indicated above, has not been shown to be of value in reducing the incidence of cholera

BACTERIOPHAGE AS A CURATIVE AGENT

On a perusal of the registers for the 'phaged villages it is found that alternate cases were treated with bacteriophage and with prodiarrhoea mixture, irrespective of the condition of the patient at the time of coming under observation In the non-'phaged villages prodiarrhoea mixture was used for the treatment of all cases From the time of starting intensive operations all cases that occurred were traced out and treated in the case of both groups of villages Taking the 31 attacks and 2 deaths of the 'phaged villages in class III they may be divided into those treated with bacteriophage and those with prodiarrhoea mixture

BACTERIOPHAGE			PRODIARRHOEA MIXTURE		
Attacks	Deaths	Case mortality rate per cent	Attacks	Deaths	Case mortality rate per cent
17	1	5.9	14	1	7.1

Bacteriophage has a slightly smaller case mortality rate But the figures are very small Somewhat larger figures are available regarding treatment of alternate cases with bacteriophage and prodiarrhoea mixture if we take figures from other

villages, which have not been included in this report   These villages had their epidemic of cholera at the same time as the experimental villages and are in the same North Arcot district, though they were outside the range of the present experiment   We are now concerned only with the relative values of bacteriophage and prodiarrhœa mixture as curative agents against cholera   Hence, as selection of cases for either form of treatment was avoided, there is no objection to the addition of these figures to the ones given above for the 'phaged villages   The combined figures are shown below —

BACTERIOPHAGE			PRODIARRHŒA MIXTURE		
Attacks	Deaths	Case mortality rate	Attacks	Deaths	Case mortality rate
36	5	13 9	33	3	9 1

Here, prodiarrhœa mixture is seen to have a slight advantage over bacteriophage

Even the combined figures are small   This has to be remembered in regard to the discussion that follows —

TABLE V

Curative treatment with bacteriophage and prodiarrhœa mixture

	Number of survivals	Number of deaths	TOTALS
Bacteriophage	31	5	36
Prodiarrhœa mixture	30	3	33
TOTALS	61	8	69

$$\chi^2 = 0\ 414 \qquad P = 0\ 520$$

The value of P makes it possible that the difference may be solely due to chance   On the available data there is no reason to think that either bacteriophage or prodiarrhœa mixture has a decided advantage over the other

The application of χ^2 test in this case and the ones that follow may not be fully justified because the figures in some cells are small   We are, therefore, applying another test as well.

Second test —On the assumption that the 36 attacks among those treated with bacteriophage and the 33 attacks among those treated with prodiarrhœa mixture are two random samples from the same population we may inquire whether the difference in the rates of case mortality falls within the range of fluctuations of sampling

Bacteriophage—

Number of attacks	36 (n_1)
Number of deaths	5 (t_1)

Prodiarrhœa mixture—

Number of attacks	33 (n_2)
Number of deaths	3 (t_2)

Let $d = \frac{5}{36} - \frac{3}{33} = 0\ 0480$

Our best estimate of p is $\frac{5+3}{36+33} = 0\ 1159$

$q = 0\ 8841$

Standard error of d or $\sigma d = \sqrt{0\ 1159 \times 0\ 8841 \left(\frac{1}{36} + \frac{1}{33}\right)} = 0\ 0771$

$\frac{d}{\sigma d} = \frac{0\ 0480}{0\ 0771} = 0\ 623$

The distribution of d in repeated sampling is likely to be far from normal when the samples are small (36 and 33)   Let us find the shape of the distribution of d —

$$\beta_1 = \frac{1-4pq}{pq} \times \frac{(n_1 - n_2)^2}{(n_1 + n_2)\ n_1 n_2} = 0\ 0006$$

$$\beta_2 = 3 + \frac{1-6\ pq}{pq} \times \frac{n_1^2 - n_1 n_2 + n_2^2}{(n_1 + n_2)\ n_1 n_2} = 3\ 0549$$

There is, therefore, very little deviation from symmetry though the curve is slightly more peaked than in the case of the normal distribution ($\beta_1 = 0$, $\beta_2 = 3$)

In any case, with the value of $\frac{d}{\sigma d} = 0\ 623$, the difference in the rates cannot be significant   On the available figures, no significant difference has been shown between bacteriophage and prodiarrhœa mixture as curative agents against cholera

RATES OF MORTALITY IN THE 'PHAGED AND CONTROL VILLAGES

Before discussing the mortality rates certain points may be mentioned   The village headman who is responsible for the registration of vital statistics is, as is well known, indifferent to his task and the figures relating to class I, that is before the arrival of the health staff, are, under normal conditions, likely to consist mostly of the severe cases which ended in death   Deaths do not readily escape notice and are, therefore, duly recorded while mild cases are easily lost sight of   Under such circumstances, the case mortality rate for the period is likely to be very high   In regard to these experimental villages all previous cases were traced by house to house enquiry and brought on record   In dividing attacks and deaths into the four classes, deaths which related to the attacks of the previous class were transferred to that class

It may be seen that, the case mortality rates for class I of both the 'phaged and non-'phaged villages are similar, 50 to 55 per cent   If the milder cases were not traced the case mortality rate would have stood very much higher

As an epidemic of cholera is characterized by varying rates of attack and mortality during the different phases of its prevalence it is necessary to examine the figures for the respective classes separately   Tables VI and VII set out the figures and the rates   In Table VI the figures for the 'phaged villages relate to group II ('phaged only) and in Table VII to the combined figures of groups II and IV, on the assumption that small doses of bacteriophage might have spread from class II to the members of class IV   As regards the non-'phaged villages, it is only figures of group II that have been used in both tables   Thus, all the figures are exclusive of inoculation   In the control villages the number of persons protected by inoculation was over two times greater than in the case of the 'phaged ones (682 against 329)

TABLE VI

Comparison of the rates of attack and mortality of the populations of the 'phaged and non-'phaged villages

Classes	'PHAGED VILLAGES (GROUP II)						NON 'PHAGED VILLAGES (GROUP II)					
	Popula tion at risk	A*	D*	Attack rate per mille	Death rate per mille	Case mor tality rate as a per centage of attack	Popula tion at risk	A*	D*	Attack rate per mille	Death rate per mille	Case morta lity rate as a percen tage of attack
I	1 239	36	19	29 1	15 3	52 8	1,592	22	12	13 8	7 5	54 5
II	1,330	48	15	36 1	11 3	31 3	1,187	28	12	23 6	10 1	42 9
III	1,475	22	2	14 9	1 4	9 1	1,855	33	15	17 8	8 1	45 5
IV	1,464	19	2	13 0	1 4	10 5	1,859	28	12	15 1	6 5	42 9

* A represents attacks and D deaths

<center>TABLE VII</center>

Comparison of the rates of attack and mortality of the populations of the 'phaged and non-'phaged villages

Classes	'Phaged villages (groups II and IV)						Non-'phaged villages (group II)					
	Population at risk	A*	D*	Attack rate per mille	Death rate per mille	Case mortality rate as a percentage of attacks	Population at risk	A*	D*	Attack rate per mille	Death rate per mille	Case mortality rate as a percentage of attacks
I	1,239	36	19	29 1	15 3	52 8	1,592	22	12	13 8	7 5	54 5
II	1,330	48	15	36 1	11 3	31 3	1,187	28	12	23 6	10 1	42 9
III	1,782	29	2	14 9	1 1	6 9	1,855	33	15	17 8	8 1	45 5
IV	1,782	26	2	13 0	1 1	7 7	1,857	28	12	15 1	6 5	42 9

<center>* A represents attacks and D deaths</center>

On a comparison of the figures for class III of the 'phaged and non-'phaged villages it is seen from both the Tables VI and VII, that the general and case mortality rates are higher in the case of the non-'phaged villages than in the 'phaged ones There is not the same marked difference between the attack rates and we have seen, by the tests carried out for judging the prophylactic value of bacteriophage, that the difference in the attack rates is not significant

<center>CASE MORTALITY</center>

<center>TABLE VIII</center>

Comparison of case mortality in prophylactically 'phaged and non-'phaged villages

	Number of survivals	Number of deaths	TOTALS
Non-'phaged	16	12	28
'Phaged	17	2	19
TOTALS	33	14	47

$$\chi^2 = 5\ 657 \qquad P = 0\ 017$$

This value of P shows that the difference in the rates is significant   The reduction in case mortality rate in the case of the 'phaged villages as compared with the non-'phaged ones seems to be real and not due to chance

Second test —

d or difference in the rates of mortality $= 0\ 3233$

$$\sigma d = 0\ 1359$$

$$\frac{d}{\sigma d} = 2\ 379$$

$$\beta_1 = 0\ 0025$$

$$\beta_2 = 2\ 986$$

With the above values of β_1 and β_2 the difference in mortality rates may be real and not due to chance

MORTALITY RATE OF THE POPULATIONS AT RISK

Two comparisons may be instituted to bring out the difference in the rates of mortality between the prophylactically 'phaged and the non-'phaged populations —

(1) A comparison of the mortality rate of group II of the 'phaged villages may be made with that of the ' not inoculated ' population of the control villages   Both figures are exclusive of inoculation

TABLE IX

Mortality rates of 'phaged and non-'phaged populations

	Number of survivals	Number of deaths	TOTALS
'Phaged	1,462	2	1,464
Non-'phaged	1,847	12	1,859
TOTALS	3,309	14	3,323

$$\chi^2 = 5\ 056 \qquad P = 0\ 025$$

If $P = 0\ 05$ is taken as the significance level the difference in the mortality rates is significant

Second test —

$$d = 0\ 0051$$

$$\sigma d = 0\ 0023$$

$$\frac{d}{\sigma d} = 2\ 22$$

$$\beta_1 = 0\ 004$$

$$\beta_2 = 3\ 070$$

These values suggest that the difference in rates may be real

(2) A comparison of the rate of mortality in the combined populations of groups II and IV of the 'phaged villages with that of the ' not inoculated ' population of the control villages   The populations of groups II and IV of 'phaged villages are combined on the assumption that bacteriophage might have spread from the members of group II to group IV   The figures are exclusive of inoculation

<div align="center">TABLE X</div>

<div align="center">*Mortality rates of 'phaged and non'-phaged populations*</div>

	Number of survivals	Number of deaths	TOTALS
'Phaged	1,780	2	1,782
Non-'phaged	1,847	12	1,859
TOTALS	3,627	14	3,641

<div align="center">$\chi^2 = 6\ 755$ $P = 0\ 009$</div>

The value of P is significant   The difference in the rates of mortality seems to be real and not due to chance '

Second test —

$$d = 0\ 0053$$
$$\sigma d = 0\ 0020$$
$$\frac{d}{\sigma d} = 2\ 65$$
$$\beta_1 = 0\ 00013$$
$$\beta_2 = 3\ 071$$

Therefore, the difference in the mortality rates is significant

We have to remember that the figures are small but, on the available data, the fall in the mortality rate in the case of the 'phaged population seems to be real and not due to chance   We have seen that, as regards class III, every case received treatment either with prodiarrhœa mixture or with bacteriophage in the case of both groups of villages   We have also seen that, as far as the available data indicate, neither prodiarrhœa mixture nor bacteriophage has a decided advantage over the other as a curative agent against cholera   To what, then, should the reduction in the mortality rates of the 'phaged villages be attributed ?

Both the death rate per mille and the case mortality rate show a significant fall, as regards class III, in the case of the 'phaged villages as against the non-'phaged ones   The former rate is the combined result of the attack rate and the case mortality rate   I have taken the figures of case mortality for the discussion that follows.   The fall in the rate might have been due to more than one cause

(1) The villages in the 'phaged group were, as regards class III, at a later phase of the cholera epidemic prevalent in them than in the case of the non-'phaged villages A comparison of the mean periods of prevalence of the epidemic corresponding to each class brings out this point

TABLE XI

Mean period of prevalence of the epidemic in the 'phaged and non-'phaged villages

Classes	'Phaged villages	Non 'phaged villages
I	4 1 days	1 5 days
II	10 7 ,,	4 5 ,,
III	12 9 ,,	12 5 ,,

As regards class III the periods of prevalence are about the same in the case of both groups of villages while, as regards classes I and II, they are definitely greater in the case of the 'phaged villages

Can the difference in the rates of mortality between the 'phaged and non-'phaged villages be due to the fact that, in the case of the former, class III coincided with a later phase of the epidemic prevalent in them than in the case of the non-'phaged ones ?

This is discussed at length later

(2) Apart from this, if any forces making for a lessened mortality had been acting in the case of the 'phaged villages even before the advent of bacteriophage, the significant difference noted between the two groups of villages in class III might represent only the continued operation of these forces   A reference to Tables VI and VII shows that the case mortality rate of the non-'phaged villages continued to be high throughout the whole period while, in the case of the 'phaged ones, the fall was quite definite even on passing from class I to class II   Is the difference in the rates of the two groups of villages in class II significant ?

TABLE XII

Comparison of the case mortality rates of 'phaged and non-'phaged villages   Figures of class II

	Number of survivals	Number of deaths	TOTALS
'Phaged villages	33	15	48
Non 'phaged villages	16	12	28
TOTALS	19	27	76

$$\chi^2 = 1\ 040 \qquad P = 0\ 308$$

There is no significant difference between the rates of the two groups However, the possibility of the emergence of a significant difference in class III from the continued operation of the causes influencing a lessened mortality in the 'phaged villages in class II cannot be excluded

The division into classes I and II, based as it was on the date of first visit of the health staff, was quite arbitrary There are instances of villages having no class II at all and of others having no class I Therefore the experience of the two periods may be combined, excluding figures for inoculation

TABLE XIII

Combined figures of classes I and II of both the 'phaged and non-'phaged villages

Classes	'PHAGED VILLAGES						NON 'PHAGED VILLAGES					
	Population at risk	A*	D*	Attack rate per mille	Death rate per mille	Case mortality per cent	Population at risk	A*	D*	Attack rate per mille	Death rate per mille	Case mortality per cent
I and II	2,569	84	34	32 7	13 2	40 5	2,779	50	24	18 0	8 6	48
III	1,475	22	2	14 9	1 4	9 1	1,855	33	14	17 8	7 5	42 4

* A represents attacks and D deaths

TABLE XIV

Comparison of the rates of case mortality for classes I and II in the 'phaged and non-'phaged villages (combined figures of classes I and II)

	Number of survivals	Number of deaths	TOTALS
'Phaged villages	50	34	84
Non 'phaged villages	26	24	50
TOTALS	76	58	134

$$\chi^2 = 0\ 723 \qquad P = 0\ 395$$

The difference in the rates is not significant This is what would have been expected because the case mortality rates in the two groups of villages approximate much more to each other in class I than in class II and, therefore, the combination

of the figures of the two classes tends to obliterate rather than accentuate the difference in class II

From Table XIII it may be seen that the attack rate, in the case of classes I and II, was definitely higher in the case of the 'phaged villages than in the case of the non-'phaged ones

TABLE XV

Comparison of the rates of attack for classes I and II in the 'phaged and non-'phaged villages (combined figures of classes I and II)

	Not attacked	Attacked	TOTALS
'Phaged villages	2,485	84	2,569
Non phaged villages	2,729	50	2,779
TOTALS	5 214	134	5,348

$$\chi^2 = 11\ 817$$
$$\text{For } \chi^2 = 10\ 0, \quad P = 0\ 00157$$

Therefore for the higher value of 11 817 of χ^2 the value of P is very much less The attack rate of the 'phaged group of villages is significantly different from that of the non-'phaged group as regards the combined figures of classes I and II

'Phaged versus non-'phaged villages

Class	Attack rate	Case mortality rate
I and II	Significant difference	No significant difference
III	No significant difference	Significant difference

Table XI shows that the mean period of duration of the epidemic in the 'phaged villages, before class III was reached, was definitely higher than in the case of the non-'phaged villages   If we make the assumption that the attack and case mortality rates are functions of the period of prevalence of the epidemic in both groups of villages the results shown above become understandable   The attack rates of the 'phaged and non-'phaged villages, which started with a significant difference, tended to approximate to each other by the time the bacteriophage operations started while the mortality rates, which at the beginning showed sufficient closeness to each other, diverged to such an extent as to produce a significant difference in class III   It would, therefore, appear that the results of the statistical tests carried out could be explained on the basis of the periods of intensive operations having synchronized with different phases of the epidemic in the case of the two groups of

villages The action of prophylactic bacteriophage in regard to either case incidence or case mortality may have been of no significance If bacteriophage had any prophylactic value, the fall in the attack rate should have been accelerated so as to produce a significant difference in rates between the two groups of villages in class III If 'phage was responsible for reducing the mortality rate in class III of the 'phaged villages as against the non- 'phaged ones, the rates of Table VI (death rate per mille and of case mortality) should have been lower than those of Table VII, because the members of group II alone received effective doses of 'phage It may be seen that this is not so

No definite answer has been given to the question as to what the cause was of the lessened mortality in the case of the 'phaged population There is a suggestion that it might be due to the fact that class III coincided, in the 'phaged and non-'phaged villages, with different phases of the cholera epidemic prevalent in them

DISCUSSION

It has to be emphasized that the figures dealt with in this report are small and that, therefore, it is unsafe to draw definite conclusions. The figures have been carefully checked and are reliable From the experience that we have had hitherto, it has been brought home to us that field experiments with bacteriophage bristle with difficulties especially when 'phaging by oral administration has to be carried out from day to day in villages, whose water-supply is not from properly constructed wells Further, constant and vigilant inspection of the work is essential if the experiments are to be properly conducted and the records correctly maintained Unless an adequate and well-trained staff is available the data collected will not be reliable Under the circumstances large scale experiments are difficult both from the administrative and financial points of view It would, therefore, appear that, even in subsequent experiments, only small figures may continue to be available However, if bacteriophage is at all useful in combating cholera, the results of repeated series of experiments should show a definite trend, though the figures for any one set of experiments might prove inconclusive

Lastly, I would again invite attention to what I urged from the very beginning of these experiments in August 1933 in West and East Godavari In field experiments there are numerous variable factors wholly beyond our control Why should we add to them by introducing inoculation as another variable factor in our 'phaged and control villages ? It was stated at the time (August 1933) that it was enough for us to ensure that about the same proportion of the populations in the 'phaged and control villages was inoculated during the course of the epidemic In my opinion this would not suffice In a village of about 350 population the Health staff might, on the day of first visit, inoculate all the available persons, about 250 or 260 In another village, with a population of 2,000, the inoculation would be spread over ten or twelve days in daily numbers ranging from a hundred to a hundred and fifty Cases like this did occur in the Godavari districts, though not in North Arcot Could it be claimed that the position of the two villages was the same ? If anti-cholera inoculation is effective against cholera and, if the prevalence of an epidemic is dependent on the upsetting of the balance between the amounts of infection and protection present in the community, the condition of the former village, with a large proportion of its population inoculated in a single day, possibly

early in the epidemic, must be different from that of the latter   Under the conditions in which these experiments must be conducted in the villages, it is not possible to avoid the occurrence of what has been stated above   I have already mentioned that large scale experiments are impossible both from the standpoint of adequate supervision and of funds   At present, the District Health Officer has, along with his other duties, to carry out the supervision of these experiments   The idea, therefore, of being able to secure a large number of experimental villages during the same cholera epidemic, so as to smooth the variations due to differences in inoculation, is to my mind not likely to be realized   I do not think it safe to combine the figures of different cholera epidemics, because it will, I think, be an attempt to combine dissimilar experiences   I would, therefore, urge that the experiments be carried out in three groups of villages, (1) a group in which only sanitary measures would be carried out, (2) another in which these measures would be combined with inoculation and (3) a third in which sanitary measures would be accompanied by the administration of bacteriophage, to the exclusion of inoculation   Such a series of experiments would yield us data by which to measure separately the value of bacteriophage and of anti-cholera inoculation as well as to gauge the relative value of the two in combating cholera

The field experiments with bacteriophage were started in West and East Godavari in August 1933 by Major A M V Hesterlow, I M S , who was then acting as the Director of Public Health, in close collaboration with Lieut -Colonel H H King, I M S , Director, King Institute, Guindy   At their request the Indian Research Fund Association made a grant of Rs 2,000 for these experiments   As the work in the Godavari districts was found to be unsatisfactory, the experiments were stopped and practically no portion of the grant was spent in the Godavari districts

Work was again started in North Arcot in November by Lieut -Colonel Webb, the Director of Public Health, on his return from leave, in consultation with Lieut -Colonel King who continued, throughout, to help the Public Health Department with his advice and guidance

I have also to mention that Mr C N Nayagam, the Statistical Assistant of this Department, was throughout associated with me in the compilation of these figures from the registers and the preparation of this report

CONCLUSIONS

On the figures available the following conclusions are reached —

1   Prophylactic administration of bacteriophage in the manner indicated above has not been shown to be effective in reducing the rate of attack from cholera

2   A reduction in the rate of mortality of the 'phaged as against the control villages is noted   There is a reduction in the case mortality rate as well as in that of the populations at risk

For reasons discussed at length, the fall in the rates has not been definitely ascribed to any particular cause   There is a suggestion that, as class III related to different phases of the epidemic in the case of the 'phaged and non-'phaged villages, this fact might explain the fall in the mortality rates

3   As regards the value of bacteriophage and of prodiarrhoea mixture as curative agents it has not been shown that the one is more useful than the other

STATEMENT

Experimental administration of cholera

Name of village	Periods of prevalence (in days)	CLASSIFICATION OF ATTACKS AND DEATHS											
		TOTAL GROUPS I TO IV			GROUP I AMONG THE 'PHACED AND INOCULATED			GROUP II AMONG THE 'IHAGED ONLY			GROUP III AMONG THE INOCULATED ONLY		
		A*	D*	Death rate per cent of attack	A*	D*	Death rate per cent of attack	A*	D*	Death rate per cent of attack	A*	D*	Death rate per cent of attack
1 *Melvaidyanam Kuppam*—													
Class I													
„ II	16	10	3	30									
Classes III and IV	11	4	1	25				4	1	25			–
2 *Sengali Kuppam*—													
Class I	11	4											
„ II													
„ III	24	4						4					
„ IV	14	2						2					
3 *Chinnadamal Cheruvu*—													
Class I	2	3	3	100									
„ II	9	7	3	42 9									
Classes III and IV	4	6	1	16 7				3	1	33 3	1		
4 *Tuthipet*—													
Class I	9	15	8	53 3									
„ II													
Classes III and IV	14	8						5					

* A represents attacks

bacteriophage in North Arcot District

Group IV Among not 'phaged and not inoculated			Group I The 'phaged and inoculated			Group II The 'phaged only			Group III The inoculated only			Group IV Given neither 'phage nor vaccine		
								POPULATIONS AT RISK (DAILY AVERAGES)						
A*	D*	Death rate per cent of attack	Population	Attack rate per cent	Death rate per cent	Population	Attack rate per cent	Death rate per cent	Population	Attack rate per cent	Death rate per cent	Population	Attack rate per cent	Death rate per cent
10	3	30										205	4 9	1 5
			86			195	0 02	0 005						
4												273	1 5	
						270	1 5							
						270	0 8							
3	3	100										427	0 7	0 7
7	3	43										423	1 6	0 7
2			34			337	0 9	0 3	3	33 3		184	1 1	
15	8	53 3										179	8 4	4 5
3			70			136	3 7		10			37	8 1	

and D deaths

Name of village	Periods of prevalence (in days)	Total Groups I to IV			Group I Among the 'phaged and inoculated			Group II Among the 'phaged only			Group III Among the inoculated only		
		A*	D*	Death rate per cent of attack	A*	D*	Death rate per cent of attack	A*	D*	Death rate per cent of attack	A*	D*	Death rate per cent of attack
5 *Pananthopur (hamlet of Rama naickenpet)*—													
Class I													
„ II	28	23	6	26 1							1		
Classes III and IV	7	2			1								
6 *Subbaman Kuppam*—													
Class I	7	14	8	57 1									
„ II	6	6	2	33 3									
„ III	19	6						5					
„ IV	14	5						4					
7 *Guddur (hamlet of Vadakkupet)*—													
Class I													
„ II	16	3	1	33 3									
Classes III and IV	11	1						1					
Totals—													
Class I		36	19	52 8									
„ II		49	15	30 6							1		
„ III		31	2	6 5	1			22	2	9 1	1		
„ IV		28	2	7 1	1			19	2	10 5	1		

N B —Figures in class I refer to the period before health staff visited the village, class II
of bacteriophage, and class IV of first
* A represents attacks

I—*concld*

			POPULATION AT RISK (DAILY AVERAGES)											
GROUP IV AMONG NOT 'PHAGED AND NOT INOCULATED			GROUP I THE 'PHAGED AND INOCULATED			GROUP II THE 'PHAGED ONLY			GROUP III THE INOCULATED ONLY			GROUP IV GIVEN NEITHER 'PHAGE NOR VACCINE		
A*	D*	Death rate per cent of attack	Population	Attack rate per cent	Death rate per cent	Population	Attack rate per cent	Death rate per cent	Population	Attack rate per cent	Death rate per cent	Population	Attack rate per cent	Death rate per cent
22	6	27 2							51	20		161	13 6	3 7
1			48			70			25			47	2 1	
14	8	57 1										360	3 0	2 2
6	2	33 3										352	1 7	0 6
1			26			279	1 8		2			39	2 5	
1			26			268	1 5		2			50	2 0	
3	1	33 3							25			189	1 6	0 5
			25			188	0 5							
36	19	52 8										1,239	2 9	1 2
48	15	31 3							76	1 3		1,330	3 6	1 1
7			289	0 3		1,475	1 5	0 1	40	2 5		307	2 3	
7			289	0 3		1,464	1 3	0 1	40	2 5		318	2 2	

of partial anti cholera operations,   class III of intensive operations including administration
14 days of intensive operations
and D deaths

STATEMENT II.

North Arcot District—Non-phuged villages

Name of villages	Periods of prevalence	CLASSIFICATION OF ATTACKS AND DEATHS									POPULATIONS AT RISK (DAILY AVERAGES)						Remarks
		Total Groups I and II			Group I Among Inoculated			Group II Among Uninoculated			Inoculated			Uninoculated			
		A*	D*	Death rate per cent of attacks	A*	D*	Death rate per cent of attacks	A*	D*	Death rate per cent of attacks	Population	Attack rate per cent	Death rate per cent	Population	Attack rate per cent	Death rate per cent	
1 *Vadakkupet*— Class I																	
„ II	4	1	1	100·0				1	1	100·0	77			150	0·7	0·7	
„ III	15	3	1	33·3	1			2	1	50·0	76	1·3		150	1·3	0·7	
„ IV	14	2	1	50·0				2	1	50·0	76			150	1·3	0·7	
2 *Bapanapalli (caste Hindus block,)*— Class I																	
„ II	1	3	2	66·7				3	2	66·7				127	2·3	1·6	
„ II	8	10	6	60·0				10	6	60·0				122	8·2	4·9	
„ III	28	6	3	50·0				6	3	50·0	32			82	7·3	3·7	
„ IV	14	4	1	25·0				4	1	25·0	32			86	4·7	1·2	

3 Bapanapalli (Cheri)—

Class I	1	1	100 0	1	1	100 0	235	0 4	0 4
„ II	3	1	100 0	1	1	100 0	234	1 0	1 0
Classes III and IV	4	1		1			233	0 4	

4 Vadakberi—

Class I	1	4	1	25 0	4	1	25 0	108	3 9	1 0
„ II	4	3	2	66 7	3	2	66 7	106	2 9	1 9
Classes III and IV									

5 Lubbaman Kuppam—

Class I	6	6	4	66 7	5	4	66 7	93	6 5	4 3
„ II	20	4	3	75 0	4	3	75 0	73	5 1	3 8
„ III	14	2	2	100 0	2	2	100 0	73	2 5	2 5
„ IV						20	20		

6 Sokkaravi Kuppam—

Class I	1	5	2	40 0	5	2	40 0	147	3 3	1 3
„ II	3	7	1	14 3	7	1	14 3	115	4 6	0 7
Classes III and IV	4	1	1	100 0	1	1	100 0	144	0 6	0 6

* A represents attacks and D deaths

STATEMENT II—*concld*

Name of villages	Periods of prevalence	Total Groups I and II			Group I Among inoculated			Group II Among uninoculated			Inoculated			Uninoculated			Remarks
		A*	D*	Death rate per cent of attacks	A*	D*	Death rate per cent of attacks	A*	D*	Death rate per cent of attacks	Population	Attack rate per cent	Death rate per cent	Population	Attack rate per cent	Death rate per cent	
7 *Ramanaclenpet* (*main village*) *First epidemic*—																	
Class I	3	1	1	100 0				1	1	100 0	.			716	0 1	0 1	
,, II	27	9	3	33 3				9	3	33 3	180			536	1 7	0 6	
Classes III and IV	14	3			2			1			265	0 8		451	0 2		
8 *Aranatla* (*hamlet of Kottacheri*)—																	
Class I	2	2	1	50 0				2	1	50 0				166	1 7	0 8	
,, II				::								.					
Classes III and IV	11	8	5	62 5	2	1	50 0	6	4	66 7	26	7 9	4 0	137	4 4	2 9	

9 Ramanaickenpet Second epidemic—

Class I	6						
„ II					25 0		
Classes III and IV	4	1	25 0	450	0 9	0 2	

J, MR

10 Ramanaickenpet (Hut area)—

Class I	10	5	5	20 0			
„ II	14	4	4	25 0			
„ III							
„ IV					20	17 2	0 8
					29	13 7	1 0

Totals—

	A	D				A	D					
Class I	22	12	54 5	1	20 0	22	12	54 5	257	0 7	1,592	1 4	0 8
„ II	28	12	42 9	1	25 0	28	12	12 9	682	0 6	1,187	2 4	1 0
„ III	38	13	42 1	5		33	15	45 5	692	0 14	1,855	1 8	0 8
„ IV	32	16	40 6	4		28	12	42 9	692	0 14	1,859	1 5	0 6

N B.—Figures in class I refer to the period before health staff visited the village, class II to the period of intensive operations, class III to the period of partial anti cholera operations (however, no 'phago was administered), and class IV to the period of first 14 days of intensive operations

*A represents attacks and D deaths

STATEMENT III

Number of consecutive days of administration of prophylactic 'phage just prior to the date of attack	Number of cases
1 dose	4
2 doses	4
3 ,,	3
4 ,,	2
5 ,,	2
6 ,,	1
7 ,,	1
10 ,,	1
11 ,,	1
12 ,,	2
21 ,,	1
24 ,	1
TOTAL CASES	23

THE ACTION OF ANTERIOR PITUITARY EXTRACT AND ITS EFFECT ON BLOOD SUGAR

BY

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AND

A N RATNAGIRISWARAN

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THE study of the anterior lobe of the pituitary and its secretion has come into great prominence in recent years, especially after the discovery of the presence in the gland, at certain periods, of a hormone stimulating to activity the follicles of immature ovaries   The work so far done has been confined chiefly to this sex-stimulating factor   The object of the present investigation was to study the general effects produced by the administration of the extract containing the growth-promoting hormone

All the experiments were done with cats weighing, on an average, about 2 kilos The animals were anaesthetized by injecting a quantity of urethane equivalent to 1 9 g per kilo body-weight of the animals   They were given their last feed about four hours previous to the administration of the anaesthetic and the experiments were conducted after a total starvation period of 24 hours

The extract of the anterior lobe of the pituitary used was prepared according to the method of van Dyke and Lawrence (1930) and 1 c c of the extract corresponded to 0 5 g of the desiccated gland   The extracts were given intravenously through the femoral veins of the animals   The possibility that the experimental results obtained might have been due to the presence of breakdown products of tissues was eliminated by means of controls with other tissue extracts

Effect on blood-pressure and respiration —Very small doses of the extract of anterior pituitary, e g , 0 125 c c , raised the blood-pressure to a slight extent and produced a small decrease in the amplitude of respiration at the end of the expiratory phase   With 0 5 c c of the extract, there was a fall of blood-pressure following an

initial rise and a subsequent recovery The amplitude of respiration decreased as with the smaller dose One c c of the extract produced the same effect on blood-pressure as 0 5 c c , but the respiration in this case was slower than before and its amplitude was diminished both during the expiratory and the inspiratory phases (Graph 1) Respiration almost stopped with 2 5 c c of the extract and recovery was very slow (Graph 3) After recovery, subsequent doses up to a total quantity of 13 c c to 15 c c were tolerated by the animal, provided the injections were given slowly and gradually allowing sufficient time to elapse between one injection and the next But when 1 c c of the extract was given all at once as a single dose, the blood-pressure dropped down nearly to the zero level and there was cessation of respiration though the heart was still beating A few minutes after, the blood-pressure and respiration improved and further doses of the extract given slowly and gradually produced a persistent rise of blood-pressure (Graph 2) When the animals had had more than 13 c c to 15 c c of the extract, they were seized by convulsions and died on account of respiratory failure There was no respiratory embarrassment when the extract was given after bilateral vagisection or after atropine

The spleen volume corresponded to the variations in blood-pressure The intestinal volume was similarly affected The movements of the plain muscles appeared to be inhibited to a slight extent

Effect on blood sugar —The animals were starved as before for 24 hours before the commencement of the experiments Estimations of blood sugar were done by the micro method of Folin modified by Herbert and Bourne (1931) Blood was taken from the left carotid artery, the right artery having been used for recording the blood-pressure After blood had been taken for estimation of the normal value, a quantity of extract of anterior pituitary equivalent to 6 5 c c per kilo body-weight of the cat was run slowly from a burette into the femoral vein The time taken to run in 15 c c in this way was about 45 minutes Great care had to be exercised in regard both to the correct dose and rate of flow of the extract from the burette, as an overdose or a faster rate of flow was found to result in the death of the animals due to convulsions and respiratory failure The sample of blood was taken for analysis of sugar from the left carotid artery after all the extract had been allowed to flow in The results given below are representative of a series of experiments done on a total number of 39 cats —

The value of blood sugar was found to have risen to nearly twice the normal figure after the administration of anterior pituitary extract

Number	Blood sugar before anterior pituitary in mg per 100 c c of blood	Blood sugar after anterior pituitary in mg per 100 c c of blood	Percentage of rise
3770–1	121 2	222 2	83 3
3922–26	85 0	165 0	94 0
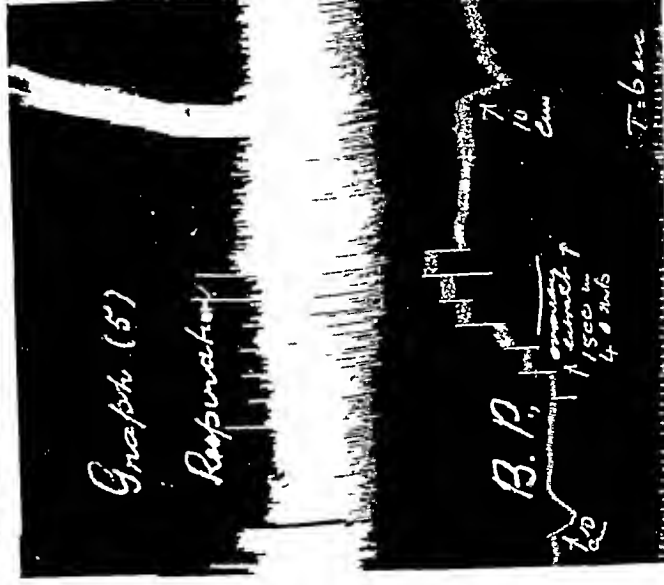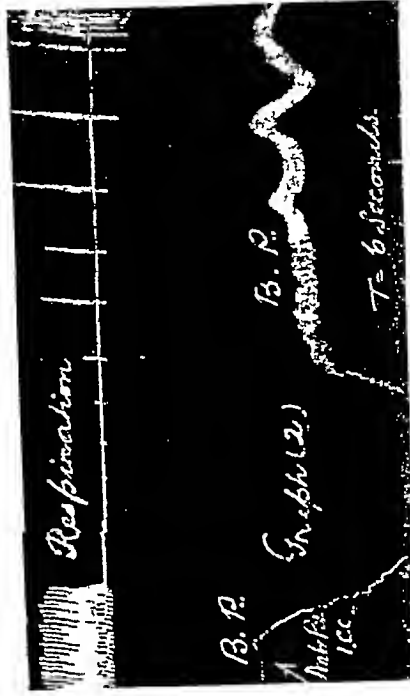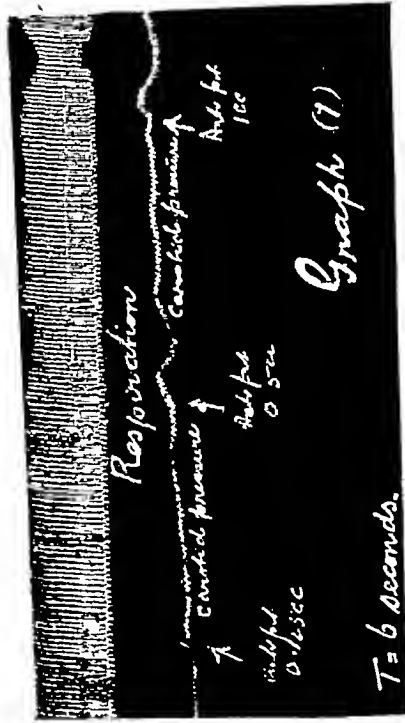
Graphs 1, 2 and 3 show the effect of injections of anterior pituitary extract on blood pressure and respiration (refer text). Graph 4 shows that the control end of the sectioned vagus requires a greater strength of current than before, the administration of anterior pituitary extract to produce the same effect on respiration and blood pressure. Graph 5—Note the increased sensitiveness of the vagal centre to electrical stimulation after injection of gonadal extract. The distances in centimetres between the primary and the secondary of the induction coil when the nerve end was stimulated are marked in Graphs 4 and 5.

Section of the vagi before administration of the extract was found to enhance the rise of blood sugar

Number	Blood sugar before vagal section in mg per 100 c c of blood	Blood sugar after vagal section in mg per 100 c c of blood	Percentage of rise
3788–90	44 0	190 0	331 8
4011–15	70 0	215 0	207 1

To determine to what extent vagal section alone could have been responsible for the increase of blood sugar, estimations were done of the blood sugar of cats with the vagi cut, but without being given the extract of anterior pituitary The results showed that there was a remarkable rise and, in some cases, the values obtained were actually greater than with the anterior pituitary alone

Number	Blood sugar before vagal section in mg per 100 c c of blood	Blood sugar after vagal section in mg per 100 c c of blood	Percentage of rise
225–8	80 0	222 0	177 5
4121–5	32 0	210 0	462 7

The increase in the blood sugar produced by the administration of anterior pituitary extract alone was not apparent when it was mixed with an equal quantity of gonadal extract of the same strength, i e , 1 c c corresponding to 0 5 g of the desiccated gland   The gonadal extracts used in these experiments were prepared by the methods described by Allan *et al* (1928) and Dodds *et al* (1930)

Number	Normal blood sugar in mg per 100 c c of blood	Blood sugar after administration of the mixture of anterior pituitary and gonadal extracts in mg per 100 c c of blood
3875–6	111 0	16C 0
3905–7	185 0	174 0

The amount of the mixture given was calculated to contain the same quantity of anterior pituitary extract as when determining its effect on the blood sugar The sex of the experimental animal should be borne in mind in choosing the gonad

as any mistake in this respect would result in a rise of blood sugar, as was the case when a mixture of orchitic and anterior pituitary extracts raised the blood sugar from 0 085 to 0 263 per cent in a female cat

Extract of the sex gland alone was found to effect an unmistakable lowering of the blood sugar that had been raised above normal by the prior administration of anterior pituitary extract

Number	Normal blood sugar in mg per 100 c c of blood	Blood sugar after administration of anterior pituitary extract in mg per 100 c c of blood	Blood sugar after later administration of gonad extract in mg per 100 c c of blood
3943–5	64 0	200 0	139 0

It was also observed that administration of gonadal extract alone reduced the blood sugar below normal

Number	Normal blood sugar in mg per 100 c c of blood	Blood sugar after gonadal extract in mg per 100 c c of blood
368–9	105 2	37 89

This fall was not noticed when the vagi were cut or after atropinization   In these cases, instead of the blood sugar diminishing there was, on the contrary, an actual rise as will be seen from the following results —

Number	Normal blood sugar in mg per 100 c c of blood	Blood sugar after vagal section and administration of gonadal extract in mg per 100 c c of blood
162–3	78 0	125 0

Number	Normal blood sugar in mg per 100 c c of blood	Blood sugar after atropinization and administration of gonadal extract in mg per 100 c c of blood
337–8	90 0	125 0

As the administration of gonadal extracts was always accompanied by convulsions and outflow of urine, blood for sugar estimation was taken long after the convulsions had ceased, to exclude possible errors on this account   If this precaution were not observed, the effect of the gonadal extract in lowering the blood sugar might easily be overlooked altogether   Further, the animals to be used for these experiments should be neither pregnant nor lactating ones, as results obtained with these were found to be invariably uncertain and unreliable

Since hypoglycæmia has been known to be associated with deficiency of adrenaline (Rabinovitch and Barden, 1932), to test whether the hypoglycæmia so produced would counteract the rise of blood sugar due to administration of anterior pituitary extract, the supra-renal veins were ligatured before giving the extract and blood analysed for sugar-content as before   As will be seen from the results cited below, instead of the expected fall there was, on the other hand, a tendency towards a rise of blood sugar

Number	Normal blood sugar in mg per 100 c c of blood	Blood sugar after ligaturing the supra renal veins and administration of anterior pituitary extract in mg per 100 c c of blood
247–8	90 0	111 0
312–3	143 0	163 0

The absence of hypoglycæmia even after ligaturing the supra-renal veins is, therefore, clear evidence that the anterior pituitary has a marked hyperglycæmic effect independent of the secretion of adrenaline

In order to find out the effects of the extracts of the anterior pituitary and the gonads on the vagal centre, the latter was tested for its response to electrical stimulation before and after the administration of the extracts   In the case of the anterior pituitary extract it was observed that the central end of the sectioned vagus required a greater strength of current to produce the same effect on blood-pressure and respiration (Graph 4)   On the contrary, gonadal extracts increased the sensitiveness of the vagal centre to electrical stimulation (Graph 5)

DISCUSSION

The correlation of the disturbances in the blood sugar values with variations in the functional activity of the anterior lobe of the pituitary is clearly seen in the clinical conditions involving the gland, such as gigantism, acromegaly, Simmonds' disease, etc   The influence of the anterior pituitary gland on blood sugar has been shown by Houssay and Biasotti (1931) who found that depancreatized toads ceased to show diabetes after removal of the anterior lobe of the hypophysis but that diabetes reappeared on transplanting the lobe or by injecting its extract   Results of a similar nature have been recorded by Houssay, Biasotti and Rietti (1932) who were able to induce symptoms of diabetes by intraperitoneal injections of anterior

pituitary lobe extracts in normal rats and dogs   The results now obtained by us by means of experiments on cats also show definitely that intravenous injections of the extract of the growth-promoting factor of the anterior pituitary produce marked hyperglycæmia   The observations of previous workers (Lepine, 1931, Emmanuel, 1931) that the actions of the anterior pituitary and the gonads were antagonistic in certain respects led us to determine the effects of the gonadal extracts on blood sugar and the results obtained showed that injections of the extracts in anæsthetized cats were accompanied by a lowering of the blood sugar below the normal

The suggestion that the effects of the anterior lobe of the pituitary on blood sugar might be due to its influence on the supra-renal glands seems to us untenable in view of our findings after ligaturing the supra-renal veins   That the effects of the extracts of the anterior lobe of the pituitary and the gonad were due more to their actions on the vagal centre seemed possible in the light of the values obtained for blood sugar after section of the vagi or atropinization   The idea that the hyperglycæmic effect of the anterior pituitary extract is due to its depressant action on the vagal centre and that the opposite effect produced by the gonadal extract on the blood sugar is the result of the stimulation of the vagal centre has found support in the experimental results relating to the excitability of the vagal centre to electrical stimulation

In further trying to determine the mode of action of the anterior pituitary on the vagal centre, we have been able to obtain indications to show that the depression of the vagal centre is brought about through the intermediation of the thyroid gland   The work is being continued and the details will be published separately

SUMMARY

1   Injections of anterior pituitary extract into cats in single doses of 1 c c, corresponding to 0 5 g of the desiccated gland, reduce the blood-pressure almost to zero and produce respiratory failure   If the quantity is given slowly and gradually, there is an initial fall of blood-pressure followed by a marked and persistent rise and the animals can survive doses up to 15 c c   If this amount is exceeded, the animals die of convulsions and respiratory failure

2   The extract increases the blood sugar to nearly twice the normal value and this rise is enhanced by vagisection or atropinization

3   Vagisection or atropinization also produces in cats a rise of blood sugar

4   Gonadal extracts reduce the blood sugar and counteract the rise due to administration of anterior pituitary extract, this effect is not observed after vagisection or atropinization

5   The vagal centre is made more sensitive to electrical stimulation by the administration of gonadal extract, whereas anterior pituitary extract has the opposite effect and it is less sensitive to electrical stimulus

6   The hyperglycæmic effect of anterior pituitary extract has been shown to be the result of its depressant action on the vagal centre

7   The mode of action of the anterior pituitary lobe on the vagal centre is under investigation and results obtained so far indicate that the depressant action of the lobe may be due to the stimulation of the thyroid gland.

We are greatly indebted to Dr A S Mannady Nayar, Professor of Biochemistry of this college, and his assistants, for valuable help in the blood sugar estimations

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THE CHEMICAL EXAMINATION OF *TYLOPHORA ASTHMATICA* AND ISOLATION OF THE ALKALOIDS TYLOPHORINE AND TYLOPHORININE

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Tylophora asthmatica, Wight et Arn, is a perennial, branching climber with long, fleshy roots, belonging to the natural order *Asclepiadaceæ* It grows wild in plains forests, and in hilly places up to an elevation of 3,000 feet above sea-level, throughout Eastern and South India, Assam, Burma and the Deccan The common English names of the plant, Indian ipecacuanha, asthmatic and emetic swallow-wort, refer to its more important medicinal properties The early European medical practitioners in this country were so convinced of its virtues that in times of shortage of the true ipecacuanha they recommended the leaves and the root of this plant as a reliable substitute for use in the treatment of dysentery It is an important drug of the Indian materia medica and is used extensively in indigenous medicine as an expectorant, emetic and anti-dysenteric

Hooper (1891) showed the presence of a crystalline alkaloid in the roots of *Tylophora asthmatica* and described some of its characteristic colour reactions but the quantity isolated by him was not sufficient for a complete analysis Brill and Wells (1917) examined the leaves of *Tylophora brevipes,* an allied species indigenous to the Philippines, and reported the occurrence of an alkaloid resembling very closely the one previously found by Hooper in the roots of *Tylophora asthmatica,* but, apparently due to lack of material, they too were unable to characterize it properly There is no other evidence in the literature of a thorough chemical examination of the plant or its constituents

In view of the reports made by different observers that the drug could be used as an effective substitute for ipecacuanha, the present investigation was undertaken

The eruptions appeared on the skin a day after exposure   The first symptoms were itching with subsequent redness   On the face, the skin became red and the eyelids and surrounding tissues were markedly swollen   There was exudation of serous fluid from the cracks that had formed on the skin   The symptoms continued for about a week and then gradually subsided   Simultaneously, desquamation occurred in the form of small scales and large flakes of dried epidermis   The condition was relieved by moist compresses and the application of the usual soothing lotions   Richards and Lynn (1934) have reported the occurrence of a dermatitis with symptoms similar to those described above due to contact with the leaves of *Ceanothus velutinus*, also an alkaloidal plant though of a different family

Experimental

The material for this investigation consisted of the root, stem and leaves of the plant and was collected from the forest area near Vandalur, which is 35 miles to the south of Madras

Alkaloid —Examination for alkaloid by extraction with Prollius mixture gave marked reactions with the usual reagents

Volatile substances —Steam distillation of 500 g of the powdered drug and extraction of the distillate with ether gave only a negligible amount of residue

Preliminary examination —In order to have an idea of the amount of material extractable by solvents, 100 g of the powdered drug were extracted in a Soxhlet's apparatus successively with petroleum ether, ether, chloroform, and 95 per cent alcohol, the residues dried at 100°C and weighed

		Per cent
Petroleum ether (40°C to 60°C) extracted		3 1
Ether	,,	1 4
Chloroform	,,	1 4
Alcohol (95 per cent)	,,	3 8

For the purpose of a systematic examination, 1 kilo of the air-dried plant material was extracted in a continuous extraction apparatus with different solvents in succession and the residues obtained with each of them were examined separately

Petroleum ether extract —Tests for alkaloid or glucoside were negative   The residue was extracted with boiling 90 per cent alcohol and filtered hot   On cooling, a gelatinous precipitate was deposited which was collected and recrystallized several times from alcohol   A final crystallization from ethyl acetate gave colourless needles melting at 80°C to 81°C   Yield 0 2 g   The substance was identified as ceryl alcohol, $C_{27}H_{56}O$ Found   C   81 4, H   14 2 , $C_{27}H_{56}O$ requires C 81 8, H   14 1   The acetyl derivative melted at 64°C

The alcoholic filtrate from above deposited a sparingly soluble substance on concentration   By treatment with petroleum ether and alcohol alternately and after recrystallizing from a mixture of alcohol and ethyl acetate slender needles were obtained, M P 192°C to 193°C   The crystals gave a typical Liebermann-Burchard reaction for phytosterols   The substance could not be examined further for lack of material.

id cooling, a dirty-white precipitate was deposited which was not enough for proper entification   It behaved like a wax as regards its solubility properties

The alcoholic filtrate was further concentrated to 500 c c , and mixed with 1 equal volume of ether   The brown syrup that came down was separated from the supernatant fluid by decantation   An aqueous solution of the syrup reduced ehling's solution quite readily and gave a phenylosazone melting at 205°C to 206°C , also gave tests for potassium and chloride   The presence of glucose and potassium iloride in the syrup was, therefore, indicated by the above reactions

The alcohol-ether mixture was evaporated to dryness and the residue, which id not give the test for alkaloid, was treated with hot water   A dark-brown resin as left undissolved   The filtered aqueous solution was treated with a 10 per cent lution of lead acetate and the precipitate and filtrate examined separately after moving lead in the usual manner   The aqueous solution obtained by decomposing the lead precipitate was concentrated and extracted with ether   Evaporation of the dried ether solution left a deep-yellow residue giving typical reactions of flavones An alcoholic solution of the substance gave a dark-green colour with alcoholic ferric chloride solution   It was coloured deep red with concentrated nitric acid, and with concentrated sulphuric acid gave an orange solution   It dissolved in aqueous sodium hydroxide to give a dark-red solution

The brown aqueous solution that remained after shaking with ether contained chiefly tannins as was indicated by the following reactions   dark-green precipitate with ferric chloride, dark-brown precipitate with potassium dichromate and a greyish-yellow precipitate with gelatine-salt reagent

The solution obtained after removing excess of lead from the filtrate of the lead acetate precipitate contained chiefly glucose and potassium chloride   No crystalline substance could be separated from the thick syrup left by evaporation under reduced pressure

Hot water extract consisted mainly of mucilage and calcium salts

Dilute hydrochloric acid extract contained calcium sulphate but gave no reaction for alkaloid

Assays for alkaloidal content —The powdered parts of the plant were analysed separately for alkaloidal content by a gravimetric method based on the U S P IX method for assay of *Belladonna* root, using as the solvent a mixture of three volumes of chloroform to one volume of ether   The results given below are the averages of three determinations   It was found that there were no appreciable differences in the alkaloidal contents of the different parts of the plant and that seasonal variations did not very much affect the yield of total alkaloids —

	Percentage content of total alkaloids
Leaf	0 21
Stem	0 24
Root	0 29
Whole plant gathered in November 1932	0 30
,, ,, February 1933	0 24
,, ,, June 1933	0 26
,, ,, December 1933	0 29

Isolation and purification of the alkaloids —Fourteen kilos of the disintegrated plant material were extracted by cold percolation with 95 per cent alcohol containing 2 per cent acetic acid till the percolate gave no reaction for alkaloid with Mayer's reagent   The alcoholic extract was nearly neutralized with ammonia and the solvent distilled off first at ordinary and then under reduced pressure   The residue was repeatedly treated with 2 per cent solution of sulphuric acid to dissolve out the alkaloids   To the filtered solution cooled in ice, ammonia was added till the mixture was only faintly acid to litmus   The filtrate from the resinous deposit was strongly cooled, made distinctly alkaline with 15 per cent ammonia and shaken with chloroform   About 3 litres of the solvent were required to extract the alkaloids completely   The chloroform extracts were washed with distilled water and the alkaloids taken out again by shaking with 2 per cent solution of sulphuric acid   The acid solution was extracted with ether to remove non-basic impurities and the alkaloids regenerated by the addition of ammonia to the cooled aqueous solution   The precipitate with the solution was shaken with chloroform   the extract washed with distilled water and dried over sodium sulphate   On distilling off the solvent under reduced pressure at a temperature not exceeding 50°C , a dark-brown precipitate was obtained (15 g)   By washing with cold acetone the colouring impurities were removed and the alkaloids remained undissolved   On filtering ... , the alkaloidal mixture was obtained in the form of a dirty-white ... (12 g)

Tylophorine —The alkaloidal mixture was dissolved in the smallest possible quantity of dry chloroform and an excess of ether (about 4 times the volume of chloroform was found necessary) added till there was no further precipitation   The light-yellow precipitate was filtered, washed with ether and dried (8 g)   The dried powder was extracted with boiling benzene (3 litres) and filtered hot   The benzene solution was then decolorized with animal charcoal and the hot filtrate concentrated under reduced pressure to a volume of 1,000 c c   On gradual cooling thin colourless plates were thrown down which were filtered off and dried on a porous plate in a dark place, M P 275°C (decomp)   A further crop of crystals, M P 273°C to 274°C , was obtained by concentrating the mother liquor   Total yield   5 0 g

The crystalline base obtained by the above described process was converted into the nitrate by digesting with a slight excess of a 5 per cent solution of nitric acid in alcohol   The mixture was warmed on the water-bath and the crystalline precipitate so produced was recrystallized from hot water when colourless, stout, rod-like crystals were formed, M P 260°C   The alkaloid was liberated from the nitrate and crystallized once again from benzene when glistening plates containing one molecule of the solvent of crystallization were obtained, M P 284°C to 285°C   Yield 4 5 g   Found   C   76 12, H   7 18, N   3 38 $C_{24}H_{27}NO_4 C_6H_6$ requires C   76 38, H   7 06, N   3 38

After drying in vacuum at 100°C over paraffin and P_2O_5, crystals of Tylophorine gave the following values   C   73 05, 73 74, H   7 00, 6 94, N   3 72, 3 48 $C_{24}H_{27}NO_4$ requires   C   73 24, H   6 92 and N   3 56   Molecular weight by the ebullioscopic method using benzene as solvent 382, 376 , calculated for $C_{24}H_{27}NO_4$ 393

Methoxyl value · Found   29 46 per cent, 30 34 per cent , calculated for 4 OMe groups 31 56 per cent

Tylophorine was easily soluble in chloroform but only sparingly so in alcohol, acetone, ethyl acetate and benzene In boiling benzene, however, it was more soluble It was practically insoluble in ether On exposure to light, as also on treatment with hot solvents, it became yellow A solution of the alkaloid in dilute sulphuric acid gave the following reactions with alkaloidal reagents Mayer s reagent yellowish-white, curdy precipitate , Kraut's reagent bright orange-red precipitate , Scheibler's reagent heavy, white precipitate , Wagner's reagent dark-brown precipitate , Silicotungstic acid white precipitate

Tylophorine hydrochloride —It was obtained in the form of colourless, shining needles by adding a concentrated solution of potassium chloride to a neutral solution of the alkaloid in dilute sulphuric acid solution The crystals were washed well with 70 per cent alcohol to remove excess of the precipitant and dried on a porous plate, M P 261°C (decomp) Recrystallization was not carried out because the substance became coloured on treatment with hot water or alcohol The air-dried crystals on drying at 100°C in vacuum over P_2O_5 lost 18 82 per cent , $C_{24}H_{27}NO_4HCl$ $5\frac{1}{2}H_2O$ requires loss of weight 18 74 per cent Found for dehydrated substance C 66 57, H 6 56, N 3 26, Cl 8 17 , $C_{24}H_{27}NO_4$ HCl requires C 67 02, H 6 57, N 3 26, and Cl 8 25

Tylophorine hydrochloride was only sparingly soluble in cold water, alcohol or acetone but more soluble in the hot solvents It was also soluble in boiling chloroform in which it gave a deep-yellow solution

Tylophorine nitrate was prepared by digesting Tylophorine with hot 5 per cent solution of nitric acid in alcohol and recrystallizing the crystalline substance so obtained from hot water It separated from concentrated solutions in short, stout needles and from dilute solutions in long, thin needles, containing $1\frac{1}{2}H_2O$, M P 260°C to 261°C (decomp) The air-dried substance lost 6 04 per cent on drying *in vacuo* at 100°C over P_2O_5 $C_{24}H_{27}NO_4HNO_3$ $1\frac{1}{2}H_2O$ requires loss of weight 5 59 per cent Found for anhydrous substance N 5 64 , $C_{24}H_{27}NO_4$ HNO_3 requires N 6 14

Tylophorine sulphate —This was prepared by dissolving the base in a slight excess of a 5 per cent alcoholic solution of sulphuric acid and adding ether to slight turbidity On keeping at a temperature of 5°C overnight, rosettes of colourless needles were obtained, M P 268°C to 269°C (decomp) It was quite readily soluble in cold water and only sparingly in alcohol or acetone

Tylophorine platinichloride was precipitated as an orange-yellow powder by adding a solution of platinichloride to a solution of Tylophorine in warm, dilute hydrochloric acid It was not soluble in dilute hydrochloric acid alcohol or acetone and could not be obtained in crystalline form On heating, it charred with frothing at 247°C to 248°C Found Pt 16 69 , $(C_{24}H_{27}NO_4)_2H_2$ Pt Cl_6 requires Pt 16 31

Tylophorinine —The ether-chloroform filtrate and washings from the precipitate of Tylophorine were shaken with dilute sulphuric acid to remove the alkaloid The combined acid solution was strongly cooled in ice and excess of 15 per cent ammonia added to precipitate the base The precipitate was dissolved by shaking with ether repeatedly till the ether solution gave no reaction for alkaloid The extracts were washed with distilled water and the ether was allowed to evaporate spontaneously

in a dark place when colourless, thin needles were deposited on sufficient concentration After repeating the process two more times, prismatic needles, melting with decomposition at 232°C to 233°C, were obtained Yield 1 6 g The crystals lost on drying at 100°C. *in vacuo* over P_2O_5 2 01 per cent which would signify the presence of $\frac{1}{2}H_2O$ of crystallization $C_{23}H_{27}NO_4 \frac{1}{2}H_2O$ requires loss of weight 2 31 per cent Found for dehydrated substance C 72 00, H 6 59, N 4 39 Calculated for $C_{23}H_{27}NO_4$ C 72 41, H 7 14, N 3 67 Found methoxyl value 24 63 per cent 3 OMe requires 24 43 per cent

Tylophorinine was sparingly soluble in cold alcohol, acetone, benzene and ethyl acetate and more soluble in the hot solvents Chloroform dissolved it quite readily and it was moderately soluble in ether when freshly precipitated On exposure to ight or on treatment with hot solvents it turned slightly coloured With alkaloidal reagents it behaved just like Tylophorine

Tylophorinine hydrochloride was deposited in the form of long, colourless needles on adding strong hydrochloric acid to a nearly neutral solution of Tylophorinine in dilute acetic acid and keeping for some time at about 10°C A concentrated solution of potassium chloride was also found to precipitate the hydrochloride in crystalline condition The crystals were filtered on a funnel with sintered glass septum, washed well with cold water and dried in air The substance became coloured on heating, sintered at 248°C, and melted with decomposition at 251°C to 252°C Found loss of weight on drying at 100°C *in vacuo* over P_2O_5 6 59 per cent $C_{23}H_{27}NO_4$ HCl $1\frac{1}{2}H_2O$ requires loss of weight 6 07 per cent Found for dehydrated substance C 65 51, H 6 65, N 3 37, Cl 9 02, $C_{23}H_{27}NO_4$ HCl requires C · 66 09, H 6 76, N 3 35, Cl · 8 48

Tylophorinine nitrate prepared from a mixture of alcohol and ether formed a cream coloured microcrystalline powder melting with decomposition at 205°C to 206°C

The optical rotations of the alkaloids could not be measured as sufficient light did not pass through the solutions for observations

Colour reactions —

	Tylophorine	Tylophorinine
Concentrated sulphuric acid	Red, violet and dirty green finally	Dark-brown, red, violet and bluish green finally
Concentrated nitric acid	Brown and then crimson	Brown and reddish brown
Concentrated hydrochloric acid	Pale violet disappearing after some time	No change
Froehde's reagent	Red, violet, green and dirty-green finally	Dark brown, red, green and violet finally
Erdmann's „	Deep-violet, leaf green and dark-brown after half an hour	Brown, dark green, bluish green and dirty-brown finally

The combustion values reported in this paper are the results of microanalyses by Dr Ing A Schoeller of Berlin.

SUMMARY

1   *Tylophora asthmatica*, Wight et Arn , has been found to contain the following constituents   Ceryl alcohol, a phytosterol melting at 192°C to 193°C , a substance of an alcoholic nature, M P 89°C to 90°C , wax, caoutchouc, resins, chlorophyll, tannin, a flavone colouring matter, glucose, potassium chloride, calcium salts and two crystalline alkaloids

2   The alkaloids have been named Tylophorine and Tylophorinine   The method of isolation and some properties of the alkaloids are described   Analytical data indicate the formula $C_{24}H_{27}NO_4$ for Tylophorine   Tylophorinine has been assigned the provisional formula $C_{23}H_{27}NO_4$.

3   The leaves, stem and root of the plant contain 0 2 per cent to 0 3 per cent of total alkaloids   The alkaloidal content does not seem to be very much affected by seasonal variations

4   The alkaloids may produce a troublesome type of dermatitis, the symptoms of which have been described

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NUCLEAR DIVISION IN MALARIAL SPOROZOITES

BY

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WHILST working on the limits of atmospheric temperature and humidity under which *Anopheles stephensi* transmits malaria, we were repeatedly puzzled by certain appearances in sporozoites seen in films from infected salivary glands, fixed by methyl alcohol and stained by Giemsa's stain These appearances suggested the occurrence of nuclear division in the sporozoite, and are shown diagrammatically in the Text-figure and in the photomicrographs in Plate XX Finally, being convinced that these appearances represented a real phenomenon and not an artefact, we kept a careful record of them

The appearances seen fall into five types, as follows —

Type 1   Normal sporozoites with a single chromatin mass

Type 2   Sporozoites showing the chromatin in process of division A much reduced tiny chromatin blob occupies the centre of the sporozoite, whilst on each side of it lies an elongated deeply staining bar of chromatin In addition there appear to be minute and hazy chromatin grains (? degenerating chromatin) in the cytoplasm

Type 3   Here the nucleus of the sporozoite is clearly dividing into two the ' dumb-bell type '

Type 4   The chromatin has divided and two clearly separated nuclei
'are visible

Type 5   One of the chromatin grains has divided again and three clearly
separated nuclei are visible

Type 1 Normal. Chromatin a single mass.

Type 2 Chromatin dividing, elongated and diffuse.

Type 3 Chromatin dividing, dumbell type

Type 4 Chromatin divided, two separate nuclei

Type 5 Chromatin divided, three separate nuclei
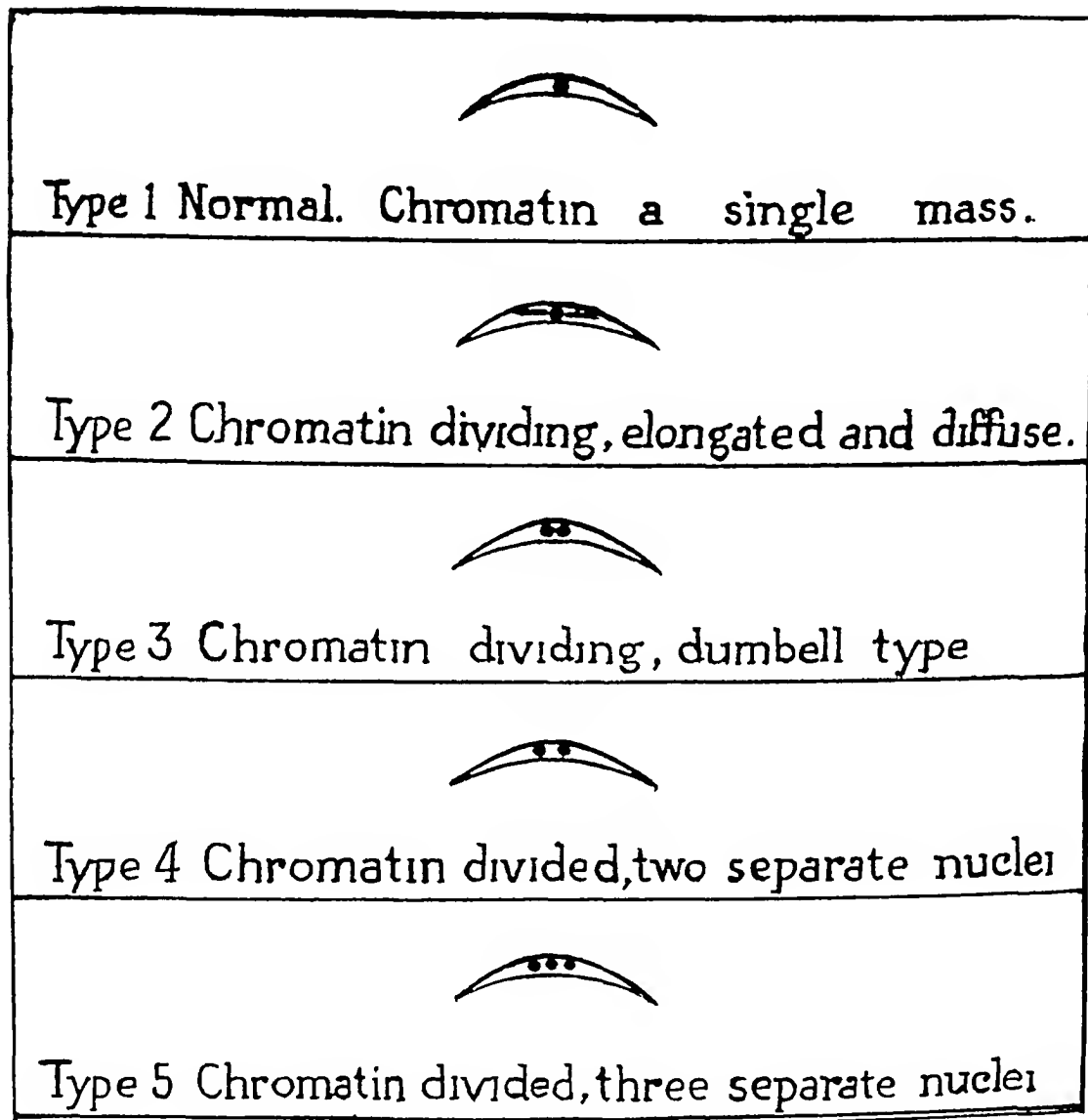
TEXT FIGURE.—Types of nuclear division in malaria sporozoites

These appearances, which were all seen in *Anopheles stephensi*, occurred
under the following circumstances —

(*i*) *Plasmodium vivax.*—A batch of *A stephensi* was fed on a patient
showing 1,120 gametocytes of *P vivax* per c mm of blood, and kept for 16 days

in the air-conditioning cabinet at 80°F and 80 per cent relative humidity The 7 survivors were dissected on the 16th day, and 6 of them showed these aberrant types of sporozoites In the stained smears the distribution of types in 718 sporozoites was as follows —

Type 1	161 or 22 per cent
Type 2	489 or 68 per cent
Type 3	30
Type 4	33
Type 5	5

(*ii*) *Plasmodium falciparum* —A batch of *A stephensi* was fed on a patient showing 320 gametocytes of *P falciparum* per c mm of blood and kept for 12 to 13 days at 80°F and 60 per cent relative humidity The 47 survivors were then dissected Of these 19 showed sporozoites with nuclear division

Two batches of *A stephensi* were fed on a patient showing 1,440 gametocytes of *P falciparum* per c mm of blood and were kept for 14 days at 80°F and 70 per cent relative humidity The 41 survivors were now dissected, and 3 of them showed sporozoites undergoing nuclear division

A batch of *A stephensi* was fed on a patient showing 1,440 gametocytes of *P falciparum* per c mm of blood and kept for 15 days at 80°F and 80 per cent relative humidity The 89 survivors were then dissected, 3 of them showed sporozoites undergoing nuclear division

A batch of *A stephensi* was fed on a patient showing 1,280 gametocytes of *P falciparum* per c mm of blood, and kept for 15 days at 80°F and 80 per cent relative humidity The 17 survivors were then dissected, 4 of them showed sporozoites undergoing nuclear division

In all, in these four experiments, 29 out of 194 infected mosquitoes (or 15 per cent) showed sporozoites undergoing nuclear division In all, 2,981 sporozoites were encountered in the films from these 29 mosquitoes, and the distribution of types was as follows —

Type 1	1,787 or 60 per cent
Type 2	1,049 or 35 per cent
Type 3	92
Type 4	53
Type 5	0

Our attention was now drawn (by Mr R Senior White, Malariologist, Bengal Nagpur Railway) to a very important paper by Missiroli (1933), which appears to explain our findings Working with *Culex fatigans* and *Plasmodium relictum* of bird malaria, this author inoculated doses of sporozoites from fresh dissections (*i*) intramuscularly, and (*ii*) subcutaneously into birds The site of injection was then excised at intervals of from 5 to 30 minutes after the injection, the tissues teased out, films prepared and stained, and then studied Sections were also studied Missiroli finds that after injection of the sporozoite its nucleus divides into 4 or 5 daughter nuclei, which are distributed along the length of the sporozoite The sporozoite itself then divides into a corresponding number of small rounded bodies consisting almost entirely of chromatin with scarcely any visible cytoplasm

These apply themselves to the surface of the red corpuscles and presumably grow up into usual ring-trophozoite forms

As far as they go, our findings are in exact accord with those of Missiroli, but ours are for human malaria parasites in *A stephensi*

DISCUSSION

The existence of more than one chromatin dot in malaria sporozoites has been described by several authors, but none of them, except Missiroli, seem to consider it a finding of importance Ross (1923) in his *Memoirs* (p 291), in a letter to Manson in 1898 describes the 'germinal rods' seen by him when working with *Culex* and *Proteosoma* as follows 'they taper towards each end and are therefore thicker in the middle In salt solution after a time they show one or more vacuoles in the middle part and become bent angularly They stain easily with Loeffler, Delafield, etc , and then show one or more chromatin granules towards the middle, between the vacuoles'

Grassi (1910) in his Plate II, figs 10 to 12, shows such multiplicity of chromatin dots He writes as follows 'the sporozoites, when almost mature or mature, by staining with the Romanowsky method, show distinct chromatin granules, situated a little apart from one another, sometimes three or even four arranged closely in rows Exceptionally, in certain cases, the sporozoites show only one nucleus, which appears to result from the fusion of the masses of chromatin'

Schaudinn (1902) in his study of *P vivax* (Plate V, figs 41 and 42) shows sporozoites with multiple chromatin masses—though the illustration is a very poor one In his Plate IV, figs 15a to 15h, he depicts the entry of a malarial sporozoite into a red blood corpuscle and its conversion into a ring-trophozoite This picture is reproduced (without comment) in Wenyon's 'Protozoology' (1926, **2**, p 914, fig 392) The senior author (R K) of the present paper has long been suspicious of the accuracy of this figure, and for that reason deliberately refrained from reproducing it in his 'Introduction to Medical Protozoology' (Knowles, 1928)

killed, embedded and serially sectioned from the 3rd to the 5th November, i e, 21 to 23 days after the infective feed. Six out of eight were found to be infected. He notes that no oocysts were present, and concludes that all had by then matured and ruptured, the mosquito's tissues (with the exception of the ovaries) were flooded with what he terms an almost veritable septicæmia of sporozoites. He makes no mention of nuclear division, however

The present paper is only by way of a preliminary note, chiefly to draw the attention of other workers to these findings. If Missiroli is right, the sporozoite may not be the end phase of the sporogony cycle. Further investigation is wanted, but it is not feasible to use man for such experiments; it may be possible to study the final end phase of the cycle in connection with bird malaria or monkey malaria

We are very greatly indebted to Lieut.-Colonel H E Shortt, i m s, Officiating Editor, *Indian Journal of Medical Research*, and to Lieut.-Colonel J A Sinton, v c, i m s, for drawing our attention to the different papers in the literature on this subject

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Note —* Indicates not seen in the original

A BLOOD-INHABITING SPIROCHÆTE OF THE GUINEA-PIG

BY

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IN a previous *Memoir* (Knowles, Das Gupta and Basu, 1932) dealing with avian spirochætosis we mentioned that we had obtained a strain of a blood-inhabiting spirochæte of the guinea-pig from Muktesar   At that time we were fully occupied with the work on avian spirochætosis, but we believe that by now sufficient data have accumulated with regard to the guinea-pig spirochæte to warrant publication

The history of the discovery of this spirochæte is as follows   In 1928 and 1929 Mr S R Hassan of the Lahore Veterinary College, whilst working on the transmission of surra, and examining the blood of guinea-pigs first encountered a naturally occurring blood-inhabiting spirochæte in this animal   He sent the strain to Mr F Ware, F R C V S, I V S, Director, Imperial Institute for Veterinary Research, Muktesar, and Mr Ware very kindly sent the strain on to us in 1930

Once the strain was received in Calcutta it was maintained in guinea-pigs by direct blood passage, one-quarter or one-half a cubic centimetre of blood was withdrawn from an infected guinea-pig at the height of the infection by cardiac puncture, and inoculated intraperitoneally into a fresh guinea-pig   The blood of all animals used was examined in thin and thick blood films before they were inoculated, and no protozoa or spirochætes were seen in any of them prior to inoculation

CLINICAL NOTES

Sixty guinea-pigs each received 1/4 c c of infected blood intraperitoneally   The infection took in all, the incubation period to the first appearance of spirochætes in films of the peripheral blood varied from 1 to 16 days, with a mean of 3 42 ± 2 330 days, in other words the range was from 1 to 6 days   After first appearance of spirochætes in the peripheral blood spirochætes were present for from 1 to 29 days with a mean of 14 2 ± 6 748 days, in other words the positive

period ranged from 7 to 21 days   The fate of these 60 guinea-pigs was as follows —

Survived   21
Died in the acute phase, with spirochætes present in the blood   12
Died within three months after the disappearance of spirochætes,
    ' after-phase ' animals   25
Killed for experimental purposes   2

Twenty-nine guinea-pigs each received 1/2 c c of infected blood intraperitoneally   Of these one proved refractory , the remaining 28 all took   The incubation period to the first appearance of spirochætes in films of the peripheral blood varied from 2 to 9 days, with a mean of 3 45 ± 1 516 days , in other words the range was from 2 to 5 days   After first appearance of spirochætes in the peripheral blood they were present for from 5 to 28 days, with a mean of 16 87 ± 5 615 days , in other words the range was from 11 to 22 days   The fate of these 29 guinea-pigs was as follows —

Proved refractory   1
Survived   16
Died in the acute phase, with spirochætes present in the blood   4
Died within three months after the disappearance of spirochætes
    ' from the blood, ' after-phase ' animals   7
Killed for experimental purposes   1

The disease in the guinea-pig is much less severe than is avian spirochætosis in the fowl   In the above figures the survivors numbered 36, as against 16 animals which died in the acute phase , in other words the mortality rate was 16 out of 52, or 31 per cent   Also, whereas the degree of infection of the blood in avian spirochætosis may be expressed as $+++$ or $++++$ that in the infected guinea-pigs can only be expressed as $+$ or $++$

The disease is a definitely febrile one in the guinea-pig   Byam and Archibald (1921) record the normal temperature of the guinea-pig as varying from 38 5°C to 39 4°C (101 3°F to 102 9°F), with an average of 38 7°C (101 7°F)   Chart 1 shows the temperature chart of an infected guinea-pig , the temperature may rise as high as 106°F during the course of the fever, and when crisis supervenes and the spirochætes disappear from the blood the temperature may fall as low as 100°F During the febrile period the animals become weak and emaciated, are reluctant to take food, and lose weight , after the spirochætes disappear from the blood recovery is rapid, and weight is soon put on again   There is sometimes hurried respiration and difficulty in breathing at the height of the infection

Chart 2 shows the temperature chart of guinea-pig No 58, which proved refractory to infection   The temperatures here recorded are within the normal limits for the guinea-pig   For reasons which will be given later, we believe that this refractoriness was due to a previous natural infection with the spirochæte Guinea-pigs Nos 57 and 58 were each inoculated with 1/2 c c of infected blood from the same donor, and the former showed spirochætes in its blood for 22 days and a temperature of 104°F , it subsequently recovered   If we take this refractoriness of No 58 to be due to immunity acquired by a previous natural infection, we may state that 1 out of 89 guinea-pigs is about the normal ratio of infection in these animals, or 1 1 per cent

CHART 1

Temperature Chart of Guinea-pig No 62
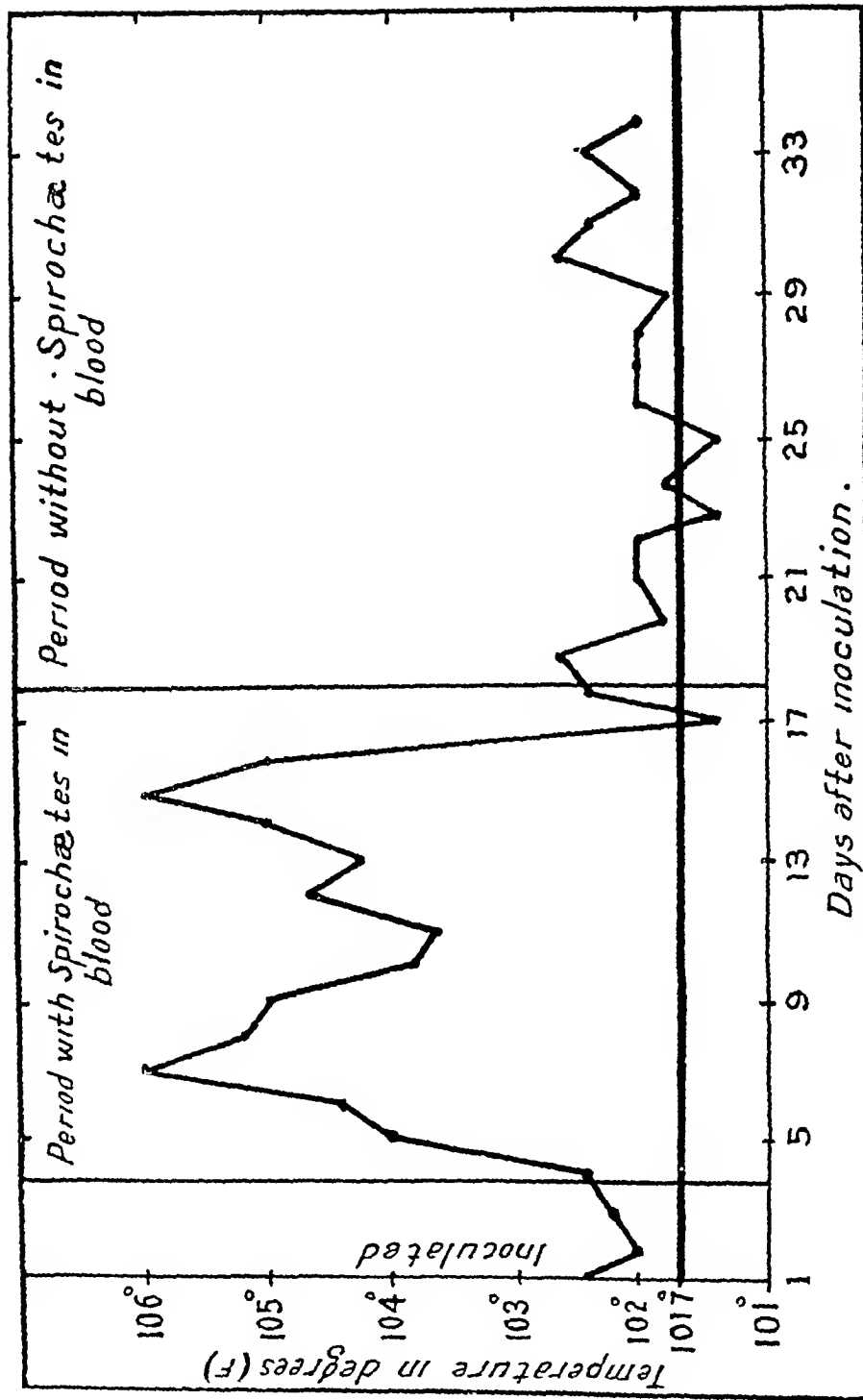
Chart 2

Temperature Chart of Guinea-pig No 58
(refractory to infection)
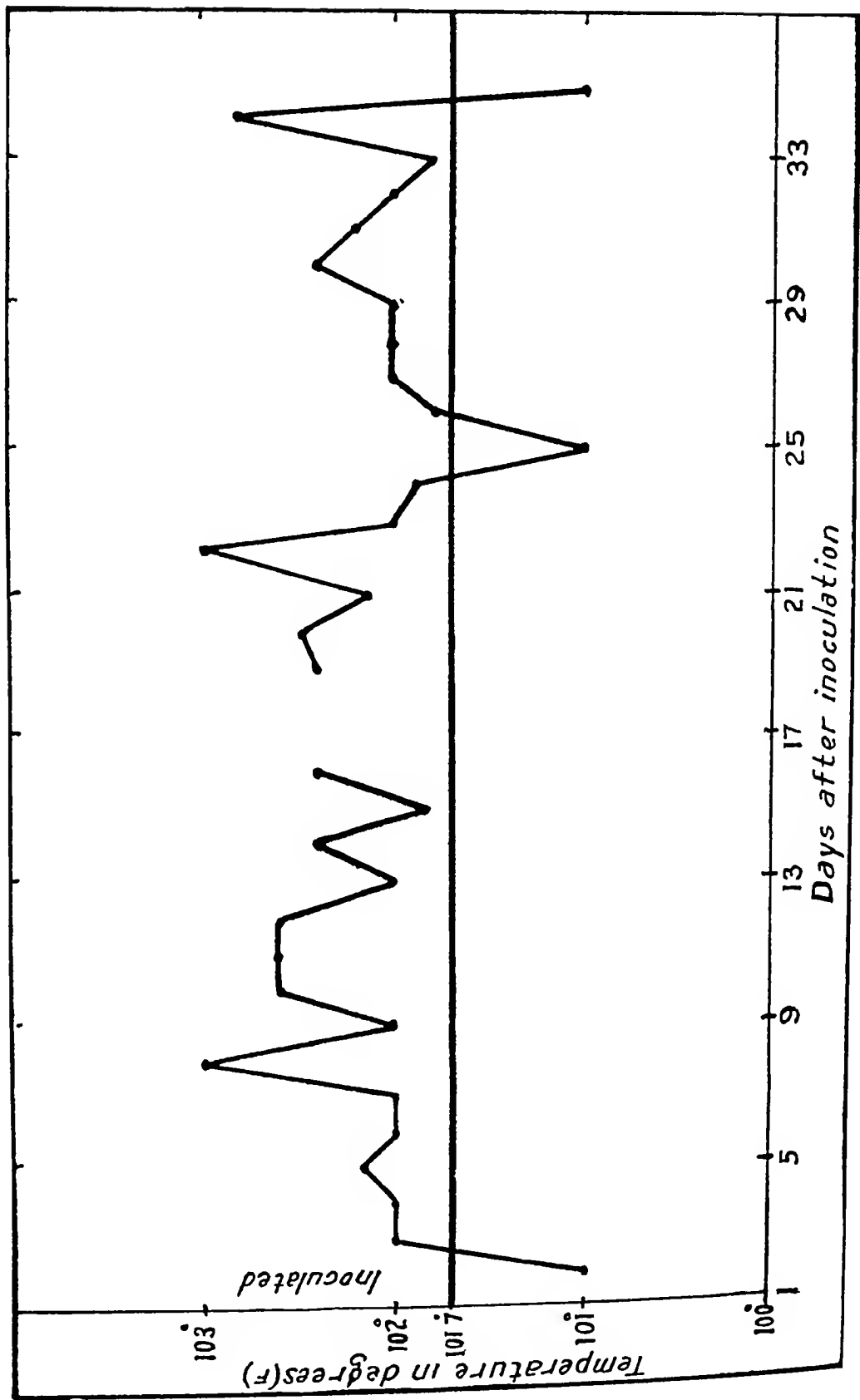
There is no tangle formation at crisis with the spirochæte of the guinea-pig, as in the case of *S anserina* The spirochætes seem to disintegrate and disappear Guinea-pigs Nos 49 and 65 were killed just after the crisis, when no spirochætes could be detected in the blood under dark-ground illumination and emulsions made of their brains, kidneys, livers, bone-marrow, spleens, lungs and testes These emulsions were examined under the dark-ground and in stained films and no spirochætes could be detected , they were also inoculated intraperitoneally into fresh guinea-pigs, but none of the latter became infected The heart blood of both animals was also searched and two fresh guinea-pigs inoculated , both the latter remained free from infection

Guinea-pig No 7 was a gravid female in late pregnancy She was inoculated with 1/4 c c of infected blood on 9th July, 1930 Spirochætes appeared in the blood on 12th July, 1930, and persisted for 21 days, till 1st August, 1930 , thereafter the blood remained free from spirochætes and the animal remained in good health under observation till the following January On 30th July, 1930, she was delivered of a litter of two at the height of the infection with the mother's blood swarming with spirochætes The blood of the two newly-born guinea-pigs was examined and no spirochætes found Two and a half months later both were inoculated with highly infected blood, and both took the infection , in one it was mild, with an incubation period of 6 days, spirochætes present in the blood for only 2 days, and subsequent recovery , in the second it was of a relapsing character, with an incubation period of 7 days, and subsequent recovery These experiments appear to show that the infection does not traverse the placenta, and that there is no hereditary transmission of the disease in the mammalian host Whether any immunity to infection is transmitted is doubtful since both the young guinea-pigs showed susceptibility to infection two and a half months after birth

Relapses were noted in 9 out of 69 animals which did not die in the acute phase, were not refractory, and were not used for experimental purposes—a relapse rate of 13 per cent Details are given in Table I —

TABLE I

Relapse cases

Serial number	Dose of infected blood given (c c)	Incuba tion period (days)	Spiro chætes + for (days)	Blood negative for (days)	Spiro chætes + for (days)	RESULT
9	¼	2	4	4	18	Survived
42	¼	3	12	4	3	,,
45	½	3	16	2	2	,,
48	½	5	15	4	3	,,
54	½	3	12	4	4	,,
65	¼	3	10	2	12	Killed two days later for ex perimental purposes
66	¼	5	10	7	2	Survived
107	¼	7	18	3	5	Died 57 days later
110	¼	3	8	6	4	Survived

In all instances there was only a single relapse   In these 9 guinea-pigs the following figures may be given as a summary of the table —

Incubation period to first appearance of spirochætes in the blood varied from 2 to 7 days , average 4 days

Spirochætes present in the blood in the first attack from 4 to 16 days , average 11 7 days

Blood then negative for from 2 to 7 days , average 4 days   Spirochætes reappeared in the blood and were present for from 2 to 18 days , average 5 9 days

Survived	7
Died 57 days after termination of the relapse	1
Killed for experimental purposes after the relapse	1

Guinea-pig No   65 was inoculated with 1/4 c c of infected blood on 20th February 1931   It showed spirochætes present from 23rd February, 1931, to 2nd March, 1931 , incubation period 3 days, first attack 8 days , then a negative phase of 2 days , then spirochætes present for 7 days , and thereafter the blood was negative for 6 days   The animal was now killed on 18th March, 1931 , emulsions were made in   normal saline of its brain, liver, kidney, lung, spleen, bone-marrow, and were examined under the dark-ground illumination and in Fontana-stained films   No spirochætes were seen   The emulsions were inoculated into clean guinea-pigs, using one animal for each organ , but results were completely negative

It is a little difficult to say what happens to the spirochætes in the negative phase between the first attack and the single relapse   Presumably spirochætes are present in the blood of the internal viscera   This point requires further investigation

The immunity resulting from an attack of the disease is of a most solid character   Two guinea-pigs were re-inoculated with heavily infected blood 15 and 20 days respectively after they had recovered from the disease , neither took the infection and both remained well for over one month whilst under observation   It is for this reason that we believe that the refractoriness of guinea-pig No   58 was due to a previous natural infection with the spirochæte

As typical examples of this infection in the guinea-pig we may quote the following —

> Guinea-pig No 6   *Mild infection with recovery* —Given 1/4 c c of infected blood intraperitoneally on 4th July, 1930   Spirochætes first appeared in the blood on 13th July, 1930—9th day   Spirochætes then present for 6 days   Maximum temperature 103°F   Thereafter the animal   remained   free   from infection   and alive and well   for 8 months, when observations were discontinued

> Guinea pig No 16   *Moderate infection with recovery* —Given 1/4 c c of infected blood intra peritoneally on 14th August, 1930   Spirochætes first appeared in scanty numbers on 16th August, 1930—the 2nd day, and were numerous (+ +) for 17 days   They then became scanty in numbers for 4 days, and then disappeared   The animal was examined from time to time for the next four months, but never showed any spirochætes and remained in good health

> Guinea pig No 20   *Death in the acute phase* —Given 1/4 c c of infected blood on 23rd August, 1930   Scanty spirochætes first present on 25th August, 1930—2nd day   These became very numerous on the 27th and 28th August, 1930, and the animal died on the latter date with its blood and organs swarming with spirochætes   Maximum temperature 106°F

Guinea pig No 110   *Relapse with recovery* —Given 1/4 c c of infected blood intraperitoneally on 19th September, 1931   Spirochætes first appeared in the blood on 22nd September, 1931—the 3rd day   The blood was then positive for 8 days   then negative for 6 days , thereafter positive for 4 days   After this the animal never showed any parasites and remained alive and well for 4 months

Virulence of the strain —The virus was continuously maintained from 17th June, 1930, to 31st October, 1931   During this period its virulence appeared to be more or less constant   Table II presents an analysis of the animals inoculated taken in historical sequence in groups of ten (animals killed for experimental purposes, animals which died in the after-phase, refractory animals, etc , are excluded)   It will be seen that in the first four series of tens, from June 1930 to January 1931 the virus was fully virulent, killing from 20 to 50 per cent of animals , then came a period from January to April 1931 (fifth and sixth series of tens) when it failed to kill , then the virulence was recovered (seventh and eighth series of tens), and when observations were discontinued the virus was killing from 30 to 60 per cent of inoculated animals

TABLE II

Virulence of passage strain

Series	Incubation period (days)	Spirochætes + (days)	Results	
			Died	Survived
First ten	1 to 9 , av 4 1	5 to 28, av 15 7	2	8
Second ten	2 to 4 , av 3 0	4 to 25, av 15 1	5	5
Third ten	2 to 5 , av 2 9	7 to 25, av 18 0	2	8
Fourth ten	3 to 5 , av 3 4	4 to 19 av 11 8	3	7
Fifth ten	3 to 9 , av 4 3	13 to 26 , av 19 0	0	10
Sixth ten	2 to 5 , av 2 9	11 to 22 av 15 9	0	10
Seventh ten	2 to 3 , av 2 8	4 to 22, av 16 2	3	7
Eighth ten	2 to 3 , av 2 4	5 to 29, av 16 8	6	4

This sudden drop in virulence is probably due to the fact that it had been preceded by a series of sub-passages which proved non-fatal in every case   The non-virulent phase was followed by inoculation into a very susceptible animal which died at the height of the infection, and the virus now recovered its virulent character   We attribute such differences in virulence as are shown rather to differences in susceptibility or resistance to the disease in different individual animals, than to any change in virulence in the strain of spirochæte

The 'after-phase'

In our *Memoir* on avian spirochætosis (*loc cit*) we have fully discussed the 'after-phase' in avian spirochætosis described by Balfour* It may be noted here that at no time during the investigation here reported on did we see any 'Balfour's granules' in the erythrocytes of the guinea-pig, though we especially searched for them Their absence we attribute to the fact that the (adult) erythrocytes of the guinea-pig are not nucleated and, therefore, the appearance of 'Balfour s granules', which we believe to be particles of chromatin extruded from the poisoned erythrocyte nuclei in the case of avian spirochætosis cannot be produced

Every guinea-pig which survived from the acute infection was kept under observation, with occasional examination of its blood for a period of at least three months after recovery—in many instances for a period of as long as six months The average life of a guinea-pig may be taken as 1,000 days, but under laboratory conditions with its blood being examined daily, and with much handling and confinement, this period must be considerably shortened Most of our guinea-pigs used were adults or young adults Hence we have concluded that any animal which survived for three months (90 days) after spirochætes had disappeared from the blood may be regarded as a survivor Those which died within three months of the disappearance of spirochætes from the blood remain to be dealt with

In all, these number 32 out of the 85 animals which survived after the disappearance of spirochætes from the blood The frequency distribution of these deaths was as follows —

Number of days between disappearance of spirochætes and death

1 day	2	16 days	1	60 days	1
2 days	2	26 ,,	1	61 ,,	1
3 ,,	3	30 ,,	1	62 ,,	1
5 ,,	1	34 ,,	1	64 ,,	1
8 ,,	1	39 ,,	1	71 ,,	1
9 ,,	1	45 ,,	1	72 ,,	1
		50 ,,	1	73 ,,	1
		53 ,,	1	74 ,,	1
		54 ,,	1	75 ,,	1
		55 ,,	1	78 ,,	1
		59 ,,	1	83 ,,	1

The first 7 of these animals may perhaps be regarded as having died from the disease poisoned by toxins resulting from the disintegration of the spirochætes, or from the effects of the disease on the tissues, but we consider that the remaining 25 deaths at from 5 to 83 days only represent the natural mortality among animals caged, and having their blood examined at very frequent intervals

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* The whole question of the after-phase and 'Balfour's granules' has been fully discussed in Knowles, Das Gupta and Basu (*loc cit*)

At the height of the infection the internal viscera swarm with spirochætes Emulsions were made from the bone-marrow, brain, liver, kidney, spleen and lung of guinea-pigs Nos 75 and 103 at the height or the infection after washing these organs in saline, and examined under the dark-ground , all were found swarming with spirochætes

Even the salivary glands are heavily infected   Guinea-pigs Nos 75 and 103 were killed at the height of the infection, the sub-maxillary and sub-lingual glands dissected out, washed well in saline to free them from blood, and emulsions made in saline   These showed numerous actively motile spirochætes under the dark-ground   One guinea-pig was inoculated with each emulsion , both took the infection , the incubation periods were 8 and 11 days respectively   spirochætes were then present in the inoculated animals' blood for 15 and 19 days respectively , they then disappeared and both animals recovered   This finding is of interest, as guinea-pigs frequently bite one another if caged together, and as the salivary glands are infected transmission by biting seems a possibility

After disappearance of spirochætes from the peripheral blood, they seem to disappear completely from the internal viscera (with the exception of course of animals which undergo a relapse   In these the spirochætes must persist in the viscera, though not seen in the peripheral blood   Unfortunately one cannot tell beforehand whether a given animal is going to undergo a relapse or not , and we have not been able to investigate the conditions here)   We may quote the following observations —

> *Guinea pig No* 49 was inoculated with infected blood on 3rd January, 1931   Its blood showed spirochætes first on 8th January, 1931 and they persisted for 15 days   They then disappeared from the blood and the animal was killed when the blood had been negative for 5 days   Emulsions were made of all the viscera and examined under the dark ground and in stained films   No spirochætes could be detected   The brain emulsion was inoculated into guinea pig No 56, and the latter animal was kept under observation for four months   It showed no spirochætes at any time

> *Guinea pig No* 65 was killed after its blood had been negative for only 2 days after disappearance of the spirochætes   Emulsions were made of its brain, kidney, liver and bone marrow   No spirochætes could be detected in the fresh emulsions, and one guinea pig was inoculated with each emulsion   None of the four animals thus inoculated showed any spirochætes at any time though all four showed a rise of temperature to 103 6°F , 104°F , 104°F , and 104 2°F respectively

The guinea-pig spirochæte is experimentally transmissible to white rats and rabbits, but not to fowls   White rat No 53 was given 1/4 c c of infected blood from guinea-pig No 49 , spirochætes appeared in the blood 2 days later, were present for 4 days , the animal's blood then cleared, but it died 4 days later   The second white rat inoculated with infected guinea-pig blood showed a relapse incubation period 3 days , spirochætes then present for 3 days, absent for 6 days, present for 3 days, absent for 4 days, present for 1 day, absent for 2 days, when the rat died   This was the only animal in the whole of the investigation which showed a second relapse

Rabbit No 50 was inoculated with infected guinea-pig blood on 10th January, 1931   Spirochætes first appeared in its blood on the 2nd day, were present for 4 days, absent for 5 days, when the animal died   Fowl No 2326 was given 1/2 c c of infected guinea-pig blood intramuscularly on 19th July, 1930   It showed no infection at any time up to its death on 10th August, 1930

The guinea-pig spirochæte would appear to be transmissible to rodents, but not to birds

Morphology of the Spirochæte

The guinea-pig spirochæte is one typical of the relapsing fever group   Under the dark-ground it shows a regular uniformity of the coils and very delicate tapering ends, and exactly resembles *S   recurrentis* and *S   anserina*   It infected blood be
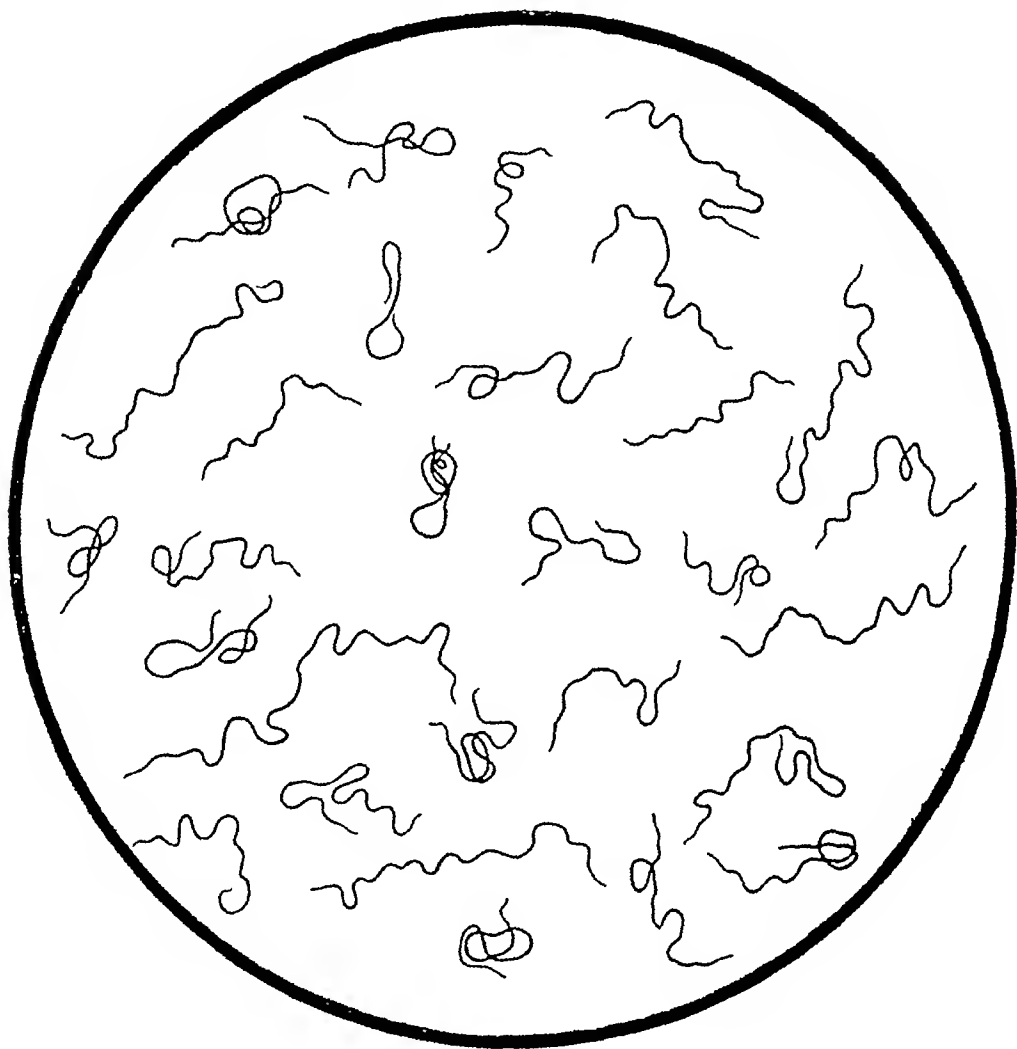
Text figure 1 —Camera lucida drawing of *Spirochæta cobayæ* showing irregular coils (occ 2, obj 1/12, Carl Zeiss)

preserved in citrated saline the spirochætes remain alive for three days, and still show uniform regularity in the coils as seen under the dark-ground   This method is also a good one for obtaining fixed and stained films of the spirochæte showing the uniform treponema-like structure   The same is true of spirochætes from culture in Galloway's medium (illustrated in Text-figure 2, and Plate XXI, fig 3)

PLATE XXI

Fig 1

Fig 2
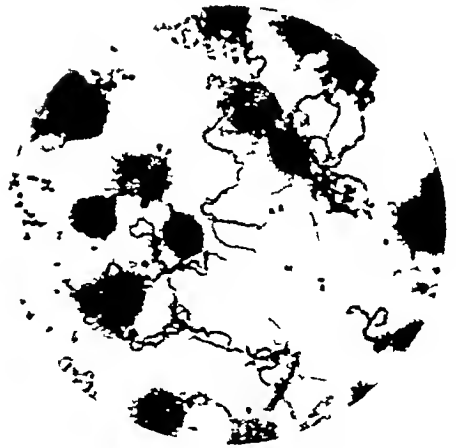
Fig 3

Fig 4

Γ gs 1 and 2 —Photomicrographs of *Spirochæta cobayæ* air dried, fixed and stained films showing irregular coils

Fig 3 —Photomicrograph of *Spiroch'a cobayæ* smear of infected blood after preserving in citrated saline showing the nniform treponema like structure

Fig 4 —Photomicrograph of *Spirochæta cobayæ* showing beaded appearance as seen in some of the infections

In air-dried, fixed and stained films, however, the spirochætes vary enormously in morphology, the coils being very irregular with loops and twists, and the appearances exactly resembling those seen in *S recurrentis* in blood films from a case of relapsing fever (illustrated in Text-figure 1, and Plate XXI, figs 1 and 2)

In order to measure the organism, blood films were taken from a guinea-pig at the height of the infection and fixed and stained by Tribondeau's modification of Fontana's stain The stained films were then placed in a microprojection
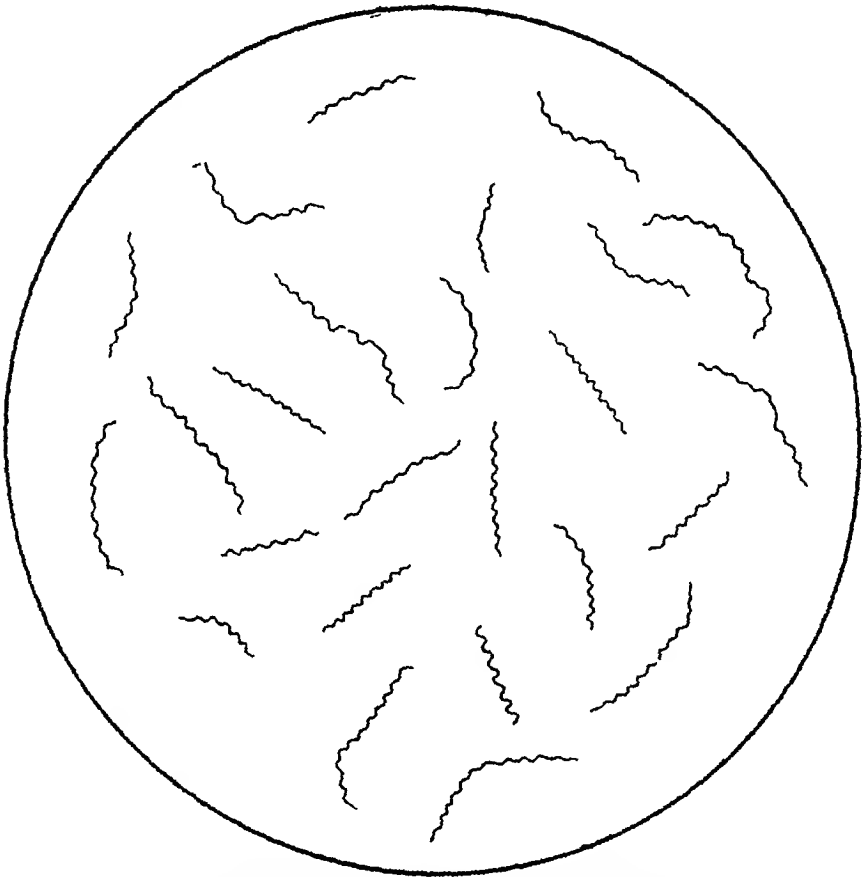
Text figure 2 —Camera lucida drawing of *Spirochæta cobayæ* showing the uniform treponema like structure (occ 2, obj 1/12, Carl Zeiss)

apparatus, and 500 drawn and measured with dividers set to one micron This gave a mean length of 18 20±4 690 microns, a mean of 5 12±1 658 coils, and a de Mello index (length/coils) of 3 6 The spirochæte is thus morphologically very like *S anserina* of the fowl, but is slightly longer and with slightly more open coils Although the two species are morphologically very much alike, they are, however, biologically profoundly different, the guinea-pig spirochæte cannot be

transmitted to fowls, whilst the avian spirochæte cannot be transmitted to guinea-pigs and rodents (Knowles, Das Gupta and Basu, *loc cit*)

Plate XXI shows the appearance of the guinea-pig spirochæte in a blood film fixed by methyl alcohol, and stained with gentian violet   When films of infected blood are spread upon grease-free slides, and fixed and stained by Nicolle and Morax's method [details of which are given in our *Memoir* (*loc cit*), (p 97)], it is
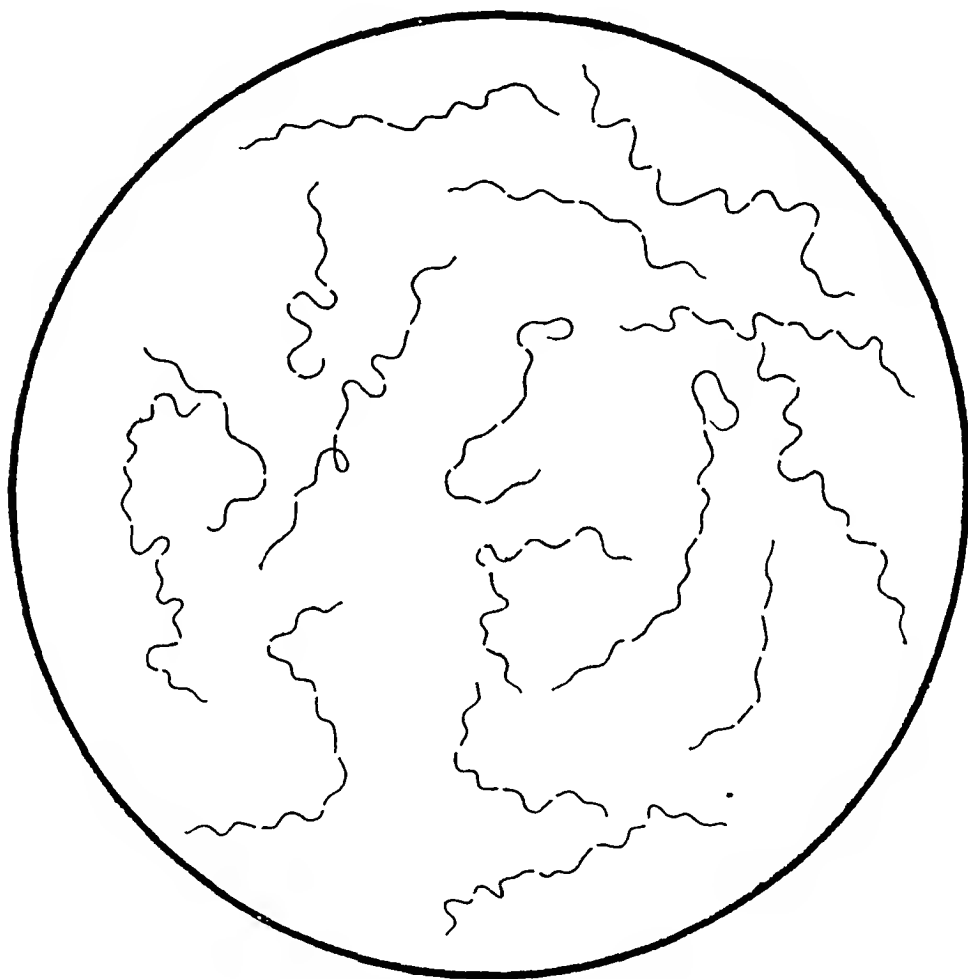
Text figure 3 —Camera lucida drawing of *Spirochæta cobayæ* showing a beaded appearance (occ 2, obj 1/12, Carl Zeiss)

seen that there is a single flagellum of great delicacy at each end of the spirochæte   Division of the spirochæte is by binary transverse fission

In some of the infections the spirochætes showed a beaded appearance as seen under the dark-ground   Colonel Acton, I M S , who saw the preparations suggested that this was due to sharp twists in the spirals, and that it was an optical effect In blood films which have been kept for some time before being fixed and stained, and which are then fixed by methyl alcohol and stained by gentian violet, a similar

appearance is seen, and is illustrated in Plate XXI, fig 4, and Text-figure 3   When viewed through light yellow filters these 'beads' in such films show up well and look like small compact rounded bodies, together with some vacuolation of the spirochæte   We do not consider that this beaded appearance indicates spore formation, but rather that it is a phenomenon of degeneration of the spirochæte

The guinea-pig spirochæte can be readily cultivated in Galloway's medium [details of which are given in our *Memoir* (*loc cit*), (p 97)]   The medium should be prepared with either rabbit or horse serum   A curious point is that growth takes place best at room temperature (27°C to 35°C), and not at body temperature   In culture the spirochæte retains its virulence for periods up to 7 to 10 days

<div align="center">TRANSMISSION</div>

Very little appears to be known about the natural ectoparasites of the guinea-pig, and accordingly we investigated the possible methods of transmission with such material as we had in hand

Lice —Sixty-four lice (*Gyropus ovalis* and *Gliricola porcelli*) were collected from three guinea-pigs who were at the height of the infection, were emulsified and the emulsions examined under the dark-ground and in stained films   Results were negative

Fleas —Four fleas collected from a guinea-pig were fed on guinea-pig No 54 when it was at the height of the infection   They were dissected three hours later   Only a few dead spirochætes were encountered in the gut contents

Bed bugs —In a series of 6 experiments a total of 17 bed bugs (species *Cimex hemiptera*) were fed on guinea-pigs at the height of the infection, and were subsequently dissected and examined at intervals of from 1 to 6 days after the feed   Results were negative

Leeches —Two leeches (*Hirundo medicinalis*) were fed on guinea pigs at the height of the infection   One was dissected 2 days later , it showed a very few motile spirochætes in the gut, crop and diverticulæ, but the rectum, ovary and salivary glands gave negative results   The second was dissected on the 16th day, and gave completely negative results

Culex fatigans —A batch of 25 laboratory-bred female *Culex fatigans* was put into a mosquito cage together with a guinea-pig at the height of the infection   Nine of these recovered from the cage at intervals of from 1 to 18 days later were dissected and examined   Scanty motile spirochætes were found in the gut contents of 6 mosquitoes up to the 5th day of the experiment, but none later   The cœlomic fluid and the salivary glands showed no infection

A batch of 15 laboratory-bred *C fatigans* was fed on a guinea-pig at the height of the infection, and re-fed 8 days later on a clean guinea-pig   No infection resulted

Anopheles stephensi and *A annularis* —A batch of 10 laboratory-bred *A stephensi* and 10 *A annularis* was put into a cage with guinea-pig No 67, when the latter was at the height of the infection   Seven of the former and 8 of the latter fed , these were removed and dissected from 1 to 8 days after the infective feed   Results were negative

DEVELOPMENT IN *Argas persicus*

As ticks appeared to be the most likely transmitting vectors, we turned to the only tick locally available in some numbers—*Argas persicus* In different experiments a total of 15 *A persicus* (11 females and 4 males) was fed on guinea-pigs at the height of the infection and kept at room temperature They were dissected at intervals of from 1 to 32 days after the infective feed It was found that development occurred in 9 (4 females and 5 males), in 3 others which were killed at the 2nd and 3rd day after feeding, the gut showed motile spirochætes, but the cycle had not had time to develop

The cycle in *A persicus*, step by step, follows that of the development of *S anserina* in this species of tick Shortly after ingestion of the infected blood the great majority of the spirochætes in the gut disintegrate and die, tangle formation is not as marked with the spirochæte of the guinea-pig as in the case of *S anserina* A few, however, survive and multiply very rapidly by binary transverse fission, long forms may be encountered where one spirochæte is dividing into several individuals By the 3rd to the 5th day abundant *tenue* forms are present These are very thin, short, and of great delicacy They are best detected under the darkground, since they are so fine and tenuous that they may easily be overlooked in stained films The cœlomic fluid becomes infected with the *tenue* forms by the 3rd to the 5th day From this every organ of the tick becomes infected In the female the ovaries may swarm with spirochætes, but we have not observed infection in the malpighian tubules In the male the white gland and testes may be invaded In both sexes the brain may be infected Invasion of the salivary glands, however, is rapid and progressive The *tenue* forms accumulate in these glands and multiply therein with great rapidity Meantime, whilst the invasion of the salivary glands is a progressive one, the infection in the gut dies out, though a very few sluggishly motile spirochætes may be found in the gut up to the 32nd day after the infective feed

The following two experiments were carried out to test the infectivity of the spirochætes in the salivary glands —

Experiment 1 Eleven *A persicus* were fed on an infected guinea pig at the height of the infection, and kept at room temperature Nineteen days later they were dissected, the salivary glands removed, washed in saline and then emulsified in saline This emulsion was then inoculated into a clean guinea pig Twelve days later spirochætes appeared in the guinea pig's blood, and were present for 13 days, they then disappeared, and the animal died 4 days later

Experiment 2 Fourteen *A persicus* were fed on a guinea pig at the height of the infection They were kept at room temperature, and dissected 12 days later The salivary glands were then dissected out, and an emulsion made and inoculated into a clean guinea pig This guinea-pig showed spirochætes first on the 10th day, they were present in the blood for 7 days, and the animal died at the height of the infection These experiments were carried out with *A persicus*, fed on guinea pigs Nos 74 and 75 (which constituted the 22nd passage of the virus by direct blood inoculation)

The spirochætes in the salivary glands are, therefore, infective to guinea-pigs Unfortunately we have not been able to secure infection by the bites of *A persicus* The following is a summary of our experiments in this matter —

Experiment 3 A total of 11 ticks fed from 7 to 12 days previously on an infected guinea pig were re fed in a clean guinea pig, No 41 Results were negative

Experiment 4　A total of 26 ticks fed from 13 to 25 days previously on an infected guinea pig were re fed on a clean guinea pig, No 76　Results were negative

Experiment 5　A total of 39 ticks fed from 8 to 31 days previously on an infected guinea-pig were re fed on a clean guinea pig, No 112　Results were negative

As we thought that *A peisicus* might feed more vigorously on the fowl than on the guinea-pig, in five experiments a total of 72 ticks fed on guinea-pigs at the height of the infection and kept at room temperature for from 8 to 19 days, were re-fed on five fowls　Results were negative

Whilst we have failed to secure actual transmission by the bite of *A peisicus*, yet the evidence given above is strongly in favour of the view that the natural infection with the spirochæte of the guinea-pig is transmitted by some tick which has access to this animal

The identity of the guinea-pig spirochæte

Only two previous workers appear to have encountered a blood-inhabiting spirochæte of the guinea-pig　Gasperi (1912) inoculated a guinea-pig subcutaneously with garden earth emulsified in a mortar with water at 85°C, in order to produce gas gangrene in it and isolate the 'vibrion septique' (*Clostridium septique*)

The animal died 20 hours later, and cultures of its heart blood were taken in glucose agar, liquefied and kept at 42°C　In these cultures he obtained not only the *Clostridium septique* but also a spirochæte　Long forms predominated in the culture, 16μ to 20μ in length, sometimes 24μ　The spirochæte was very thin and showed 5, 6 or 8 coils in a long form　The ends were fine and drawn out　Short forms were scantier than long ones, ranged from 9 6μ to 12 8μ, with 3 or 4 coils, they were relatively thick in the middle but with finely drawn out tapering extremities　Dividing forms were not seen　As seen under the dark-ground they were actively motile with a helicoid movement, progressing along a rectilineal line　The culture died out in 48 hours　The author conducted six further experiments with the same technique, but in none of these six guinea-pigs were spirochætes encountered　He concludes that the spirochæte was one occurring naturally in the guinea-pig

Macfie (1914) also cultivated a spirochæte from the heart blood of the guinea-pig　Two c c of heart blood were drawn off and placed in a sterile test-tube containing 1/10th c c of 50 per cent aqueous glucose　The blood was then defibrinated, transferred to a smaller sterile test-tube, and incubated at 37°C　On the 3rd day a few small spirochætes were found in the culture, these then rapidly increased in number　From the 79th hour, however, they disintegrated and all had disappeared by the 8th day　At first the spirochætes were very small, 2μ to 9μ in length, with 2 to 9 coils　Later, however, a few longer forms were encountered, from 13μ to 17μ in length　A sub-culture showed very minute forms, only 1μ to 3μ long　Attempts to infect a clean guinea-pig and a white rat with a culture 150 hours old had negative results　A good colour plate illustrates the article

Rhees (1932) isolated a spiral organism from the guinea-pig, but this appears to be a spirillum of *Spirillum minus* type, and not a spirochæte, to judge from her photomicrographs　Its source was not determined　In preparing culture medium for an attempt to cultivate *S minus*, a male guinea-pig was killed, its intestines with the exception of the stomach removed, and the whole body of the animal ground

in a meat grinder, the filtrate from this was used for the preparation of media Ten tubes of the medium used were inoculated with heart blood or tissue from guinea-pigs infected with *Spirillum minus* One culture became positive, the organism was from 2μ to 3μ in length, short and stumpy, with a single delicate flagellum at each end The best growth was obtained in Dorset's broth plus 0 1 per cent agar Two white mice and two guinea-pigs were inoculated with the culture but none took the infection This author appears to have obtained a culture of *Spirillum minus*, but there is no evidence that the organism in the culture was a blood-inhabiting natural spirochæte of the guinea-pig

Several authors have recorded blood-inhabiting spirochætes from small rodents other than the guinea-pig Breinl and Kinghorn (1906) found a blood-inhabiting spiral organism in the white mouse, also in the wild mouse *Mus musculus* It was a short organism however, 1 8μ to 3 8μ long, with 2 to 4 coils, and was very scanty in the blood Attempts to transmit the infection were unsuccessful No illustration is given, and, although the authors propose the name *Spirochæta laverani* nov sp for the organism, it would appear to be a spirillum, probably *Spirillum minus*, rather than a spirochæte

Nicolle (1907) described a blood-inhabiting spirochæte from the gondi, *Ctenodactylus gondi* This was present in one animal in the blood in very scanty numbers It measured from 16μ to 19μ in length, by 0 3μ in breadth It did not appear to be pathogenic and disappeared from the blood in the course of a few days The author proposes the name *Spirochæta gondii* for this organism

Mathis and Leger (1911) described a blood-inhabiting spirochæte of the rabbit *Spirochæta raillieti* sp nov This was from 14μ to 17μ in length We have not had access to the original paper, unfortunately, and can give no further details

An organism which has received a great deal of attention of recent years is *Spirochæta crocidurae* of the shrew mouse, *Crocidura stampfli*, of Dakar in West Africa This was first discovered by Leger, A (1917) Infection in the blood was scanty, about 1 spirochæte in 15 microscope fields It was actively motile and dividing forms were seen Its length was from 14μ to 16μ, width 0 25μ, with 4 to 5 coils Leger gave the new spirochæte its present name Leger and le Gallen (1917) showed that the organism is inoculable to rats, mice, field mice and monkeys, in monkeys the course of the infection resembles that of relapsing fever in man Rabbits and guinea-pigs proved refractory Later Leger (1918) remarks on the resemblance of *S crocidurae* to the relapsing fever spirochætes of man As far as he knows relapsing fever of man had not occurred in Dakar or French Senegal, but the ready inoculability of the spirochæte to mammals and monkeys suggests that the blood-inhabiting spirochætes of small mammals may play a part in the relapsing fever of man

Leger, M (1924), observed what appeared to be the same organism in *Rattus norvegicus*, *Rattus coucha*, and *Golunda campanæ* of the same locality, and again emphasizes the possibility of these rodents acting as reservoirs of the spirochæte of human relapsing fever As *Ornithodorus moubata* does not occur in this locality, some other vector must be responsible

A very interesting paper is one by Nicolle and Anderson (1927) They show that *S crocidurae* is infective to human beings, and that by all the tests applied it is closely related to the human spirochæte of African tick fever They suggest that

the relapsing fever spirochætes originated as parasites of small mammals, became adapted to human beings through the agency of ticks, and finally through their close association with lice acquired the property of survival in and transmission by these insects The consequence of the complete adaptation to the louse is that relapsing fever has spread from Africa throughout the Old World

Mathis, Durieux and Ewstifeief (1927) report cases of relapsing fever at Dakar , the strain was readily inoculable to mice, and the absence of *Ornithodorus moubata* from Dakar and the possibility of the spirochæte being *S crociduræ* suggest some other vector The same authors (Mathis, Durieux and Ewstifeief, 1927*a*) record three further cases from Dakar Dakar is clearly an endemic focus of relapsing fever and *S crociduræ* under suspicion

Nicolle and Anderson (1927, 1927*a*) show that *S crociduræ* is transmissible by *O moubata* and the infection is transmitted to the progeny The principal vectors are nymphs derived from ova laid by infected ticks Cazanove (1930) and Lasnet (1930) comment on the persistence of relapsing fever infection in Senegal and attribute this to the presence of small wild rodents, which serve as a reservoir of the virus Finally, Mathis and Durieux (1932) compared a strain of relapsing fever obtained from *Ornithodorus erraticus* found naturally infected at Dakar, with two strains from human cases of the disease, and a strain obtained from a naturally infected shrew mouse Cross-immunity experiments were made with these different strains which, though showing minor differences, confirmed the view that *O erraticus* is the transmitting host of the human relapsing fever occurring at Dakar

Table III gives a summary of the information available regarding the blood-inhabiting spirochætes of rodents

To return to the question of the identity of the blood-inhabiting spirochæte which we have described, we may rule out Rhees' organism and that described by Breinl and Kinghorn as almost certainly belonging to the genus *Spirillum*, and not *Spirochæta* Our organism corresponds fairly well to that described by Gasperi (1912), though it is rather longer , Gasperi's organism was studied only in culture, whereas ours was studied in infected guinea-pig's blood Macfic's organism from the guinea-pig was also studied only in culture , it seems to be a much shorter organism than the one which we have described, but this may be due to the differences in technique adopted Our organism does not show much morphological difference from the spirochæte of the gondi described by Nicolle (1907), from that of the rabbit described by Mathis and Leger (1911), or from *S crociduræ* of the shrew mouse discovered by Leger (1917) On the other hand morphology alone is not a safe standard to go by , even serological tests are not too trustworthy in working on blood-inhabiting spirochætes, animal susceptibility being a better test

It is very difficult to state what constitute specific characteristics in a spirochæte, and the number of synonyms in the literature is already very great We hesitate to add to confusion which is already confounded , on the other hand this blood-inhabiting spirochæte of the guinea-pig must have some name by which it may be referred to , and we suggest the name *Spirochæta cobayæ* sp nov , with the proviso that later work may possibly show that this organism is possibly a spirochæte of different species of rodents, including the guinea-pig, and that

TABLE III

Blood-inhabiting spirochætes of rodents

Author and date	Name	Natural hosts	Measurements	Animals susceptible	Transmission	Remarks
Breinl and Kinghorn (1906)	Spirochæta laverani	White mouse, Mus musculus	1 8μ to 3 8μ long, 2 to 4 coils	Experiments unsuccessful		Possibly Spirillum minus
Nicolle (1907)		Ctenodactylus gondi	16μ to 19μ long, 0 3μ wide			
Mathis and Léger (1911)	Spirochæta railleti	Rabbit	14μ to 17μ long			
Gaspon (1912)		Guinea pig	Long forms 16μ to 20μ, 5 to 8 coils. Short forms 10μ to 13μ, 3 or 4 coils			
Mache (1914)		Guinea pig	Short forms 2μ to 9μ 2 to 9 coils. Long forms 13μ to 17μ	Experiments unsuccessful		
Léger, A (1917)	Spirochæta crocidura	Crocidura stampflii, Rattus norvegicus, Rattus coucha, Golunda campanæ	14μ to 16μ long, 0 25μ wide, 4 to 5 coils	Man, field mice, rats, monkeys, mice	Ornithodorus moubata experimentally Ornithodorus erraticus in nature	
Rhees (1932)		Guinea pig	2μ to 3μ short and stumpy	Experiments unsuccessful		Possibly Spirillum minus
Present paper	Spirochæta cobayæ	Guinea pig	13 5μ to 23μ long, mean 18 2μ, Very thin Single flagellum at each end	White rats, rabbits	Develops in Argas persicus under experimental conditions	

S cobayæ may later prove to be synonymous with some previously described blood-inhabiting spirochæte of small rodents

SUMMARY

1   A blood-inhabiting spirochæte of the guinea-pig, belonging to the relapsing fever group, is described   The infection is easily transmissible from one guinea-pig to another by blood inoculation   White rats and rabbits are also susceptible

2   The course of the disease resembles that of avian spirochætosis in the fowl, but is much less severe, the mortality being about 31 per cent   After an incubation period of 2 to 6 days spirochætes appear in the blood, multiply rapidly, are present for 7 to 28 days, and then disappear   The animal may die at the height of the infection, or a few days after the spirochætes have disappeared, or may recover

3   The disease is definitely febrile in the guinea-pig (Chart 1)

4   The infection does not appear to be hereditarily transmitted, nor is there any transmitted immunity   In animals which have recovered from the infection there is a solid immunity

5   Relapses occurred in 9 out of 69 animals (Table I)

6   During the height of the infection the internal viscera swarm with spirochætes, but after the disappearance of spirochætes from the peripheral blood no spirochætes can be detected in emulsions of the internal viscera and these emulsions are not infective to clean animals

7   The spirochæte is one typical morphologically of the relapsing fever group, with a length of from 13 5 to 23 microns, thin and delicate and with finely tapering ends   By special staining a very delicate single terminal flagellum can be demonstrated at each end   The spirochæte can be readily cultivated in Galloway's medium

8   In fed *Argas persicus* the guinea-pig spirochæte develops in a manner exactly similar to that of *Spirochæta anserina*   The vast majority of the ingested spirochætes disintegrate and die   A few, however, survive and divide very rapidly   Very thin and delicate *tenue* forms are produced and these pass into the hæmocele from the 3rd to the 5th day   From this they invade all the viscera of the tick   There is heavy and progressive invasion of the salivary glands, whereas the infection in the gut slowly dies out   Emulsions of the infected salivary glands of the tick are infective on inoculation into clean guinea-pigs, but we have been unable to cause infection by the bite of infected ticks   Presumably in Nature the transmitting vector is some species of tick which gains access to guinea-pigs

9   Previous records of blood-inhabiting spirochætes of rodents are discussed (Table III)

10   It is suggested that the name *Spirochæta cobayæ* nov sp   be given to this organism

Pathologist in the Protozoology Department   We are very grateful to Mr F Ware, F R C V S , I V S , Director, Imperial Institute for Veterinary Research, Muktesar, for bringing this interesting organism to our notice and for sending us the strain

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OBSERVATIONS ON THE PATHOGENICITY OF THE LOCAL STRAINS OF *SPIRILLUM MINUS* TO GUINEA-PIGS

BY

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DIFFERENT strains of *S minus* obtained locally from human cases of rat-bite fever were seen to vary widely in their pathogenicity towards guinea-pigs   Indian authorities like Row and Pramanand (1931)[+] did not find any evidence of pathogenicity in the strains they studied   While Robertson's investigations also gave similar results, Japanese workers like Ogata, Ishiwara, Ohtawara and others reported their strains to show considerable virulence in these test animals   Knowles and Das Gupta found the Calcutta strains of the spirillum fatal to young guinea-pigs   McDermott's strain caused a progressive disease in these experimental animals which ended fatally on the 63rd day of inoculation   Sub-passages in them resulted in exaltation of virulence of these organisms, tending to show a stabilization of this property, causing death on the 23rd day as compared with the 63rd day of his original animal   The virulence towards guinea-pigs of a few local strains of the spirillum has been studied and the results thereof are embodied in the present communication

Rat-bite fever is by no means uncommon in Vizagapatam and, if the incidence of the disease in a locality can be taken as an index of the rat infection there, the infection rate must be fairly high in this place   About a dozen cases a year come up to the laboratory for diagnosis and they are scattered throughout the year, disclosing no particular seasonal incidence   A short résumé of the case history of one of the patients is added here as it presents more than one point of special interest   The circumstances of the case led to a suspicion that an associated infection with *T pallidum* may influence the virulence of the *S minus* in the direction of exaltation

Mr R , a man of 38 years, was seen with fever, anæmia and extreme weakness , his history recorded some kind of bite on the finger during sleep which healed

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* But Knowles and Das Gupta (1928) state that ' he (Pramanand) finds that the experimental disease is very fatal in guinea pigs '

spontaneously; after an interval of 15 days, fever started with rigor The maximum temperature recorded was 102°F, and the duration of fever 3 days at a time   The patient had not noticed any rash and there was no secondary flaring up of the primary scar   At the time of examination he had already had six relapses and on each occasion he had taken quinine   In addition to fever and a sense of extreme prostration, he complained of severe headache, pains all over the body and constipation   Patchy pigmentation of the palms and soles, enlargement of the groin, axillary and epitrochlear lymphatic glands and a history of penile sore some years previously suggested the probability of a pre-existing syphilitic infection   Evidence of a mild grade bronchitis, a puffy face, a pale swollen tongue, a palpable tender spleen and a short systolic sound with a bruit in the mitral area completed the picture   Dark-ground examination of the blood was negative for the spirillum and prolonged examination of a stained smear revealed only a polymorphonuclear increase and an eosinophilia to which much importance was not attached in view of the commonness of helminthic infection in these parts   The erythrocyte count was 3 5 millions per c mm, and the anæmia was of the hypochromic type   His blood was strongly positive to the Wassermann test

One c c of the patient's blood was injected intraperitoneally into a young guinea-pig   The patient was given two injections of 0 15 g and 0 3 g of N A B   All his symptoms except weakness disappeared and he was instructed to come back after one month   After the expiry of this period, during which he remained free from any trouble, his blood was again tested by the Wassermann reaction which still gave a strong positive result and he was advised to take the specific treatment

Blood from the inoculated animal was examined every other day from the 8th to the 17th day with negative results   On the 71st day definite illness in the animal attracted attention   Blood examination revealed the presence of the parasite both by the dark-ground method and in smears stained with Leishman's stain, the latter showing three to four spirilla per field   There was also marked neutrophilia   Subsequently keratitis and conjunctivitis with marked discharge appeared almost simultaneously with the appearance of a copious discharge from the nose, well-marked alopecia about the snout and œdematous infiltration of the scrotum and perianal region   The animal by this time appeared very sick, showed disinclination to move about and exhibited a general ruffling of the coat and wasting   The temperature was never more than 104°F   On the 71st day the Wassermann reaction was strongly positive   On the 75th day the blood examination again showed plenty of spirilla   As there was at this time evidence indicative of secondary septicæmia, a specimen of blood was plated on MacConkey's medium   In 24 hours numerous acid colonies of a coliform bacillus grew   Apprehending death 1 c c of blood from the animal was inoculated into two other healthy guinea-pigs, specimens of blood from which had previously been tested by the Wassermann reaction with negative results   By the 78th day the snout was completely denuded of all fur and presented small ulcers scattered about and crusted with blood clots   The serum now was very turbid and milky and a drop put in a test-tube of distilled water showed a heavy trailing cloud of globulins   The discharge from the eye and the nose continued to be copious and the animal gave an impression of blindness   It died on the 79th day

The chain of events in one of the freshly infected animals was the same except that the Wassermann test became positive so early as the 27th day, the parasites

appeared ın the perıpheral blood for the fırst tıme on the 52nd day and death occurred on the 72nd day   The other anımal dıed as early as the 29th day   The death of thıs anımal on the 29th day was probably due to other causes, although the spleen smears from ıt showed the presence of ınfectıon   It was also notıced ın the other anımal that the eye condıtıons evınced a dıstınctly relapsıng character

The straın was kept goıng through nıne   sub-passages by ınoculatıng a clean anımal every tıme the sıck one revealed evıdence of threatenıng death and a spırıllum septıcæmıa   It ıs ınterestıng to note that one of the anımals ınjected dıd not take the ınfectıon but remaıned refractory to ıt   Sub-passage from thıs was futıle   The earlıest case of death due to the spırıllum occurred on the 41st day after a contınuous ıllness wıth persıstent blood ınfectıon from the 23rd day Judgıng from these passage experıments ıt looks quıte probable that a steppıng up of vırulence of the organısm can be brought about by such passages

Durıng the course of thus preservıng the straın, the Wassermann test was done on all the anımals prevıous to ınoculatıon as well as after establıshed ınfectıon wıth posıtıve results ın the latter ın most ınstances and negatıve ın all the former except one, the blood of whıch gave a posıtıve reactıon (+)   From these experıments, therefore, ıt ıs not unreasonable to conclude that, at any rate ın the advanced cases, the occurrence of an antıbody whıch fıxes the complement ın conjunctıon wıth the usual Wassermann antıgen ıs an almost constant phenomenon ın the blood of the ınfected guınea-pıgs as agaınst the 50 per cent posıtıve results recorded ın the case of *S mınus* ınfectıon ın man

In one ınstance swabs taken from the mouth and the nose were shaken ınto normal salıne whıch was then ınjected ınto the perıtoneum of a healthy guınea-pıg Thıs latter dıd not take the ınfectıon , no evıdence of a posıtıve blood examınatıon was forthcomıng and the anımal remaıned healthy under observatıon for 6 months Three of the sıck anımals, whıch would have been destroyed ın the usual course, were each gıven ıntraperıtoneally two doses of 0 01 g and 0 02 g of N A B well dıluted ın normal salıne at ıntervals of 5 days   One dıed after the fırst ınjectıon and probably due to ıt   Though after the fırst ınjectıon the other anımals also seemed to be almost on the verge of death, they rallıed and got completely well after the second dose, repeated blood examınatıon yıeldıng no posıtıve results at any tıme thereafter and sub-passages faılıng to ınfect   In these cured cases the Wassermann reactıon remaıned posıtıve whıch, of course, could not have been due to any antecedent syphılıtıc ınoculatıon   It ıs ınterestıng to note that after a year when one of these cured guınea-pıgs was kılled and an autopsy was done all that was found of the spleen was only a very small black patch stıckıng to the posterıor abdomınal wall   The omentum was reduced to two or three nodular tags and the vıscera had extensıve adhesıons poıntıng probably to a prevıous genera- lızed perıtonıtıs   The lıver showed both macroscopıcally and mıcroscopıcally a severe grade fatty degeneratıon but ın spıte of all these effects the anımal showed no ındıcatıon of ıll health

Half a cubıc centımetre of serum from one of the dıseased anımals taken a couple of days before death was mıxed wıth an equal quantıty of cıtrated whole blood contaınıng the organısm and the mıxture was ınoculated ınto the perıtoneum of a healthy anımal after ıncubatıng for two hours   Thıs guınea-pıg dıd

not develop the disease, nor did the blood examination betray any infection Whether it was due to the destruction of the spirillum by any lytic antibodies contained in the serum or whether it was only due to the refractory nature of the animal cannot be decided on this one experiment and the question is being further studied   Anti-spirillum agglutinins have been demonstrated to be present in the serum by other workers on the subject and the immunity response in rat-bite fever seems to bear a close analogy with that in relapsing fever and syphilis

Three more strains were studied   All the three patients from whom the organisms were derived showed a positive Wassermann reaction   Whether this positive result was due to an independent spirillum infection or whether it was due to an associated syphilis could not be investigated in two of the patients as they did not return after the initial treatment   The other was a boy of 12 , his blood, taken 6 weeks after the treatment for rat-bite fever, was negative to the Wassermann test and he exhibited no symptoms of inherited lues   In the case of this patient there was only a local tenderness but no secondary flaring up of the healed wound   Both his blood and the tissue-juice from the vicinity of the scar were positive for the spirillum, those from the latter situation being markedly longer than the blood forms   An animal inoculated from his blood showed a temperature of 103°F on the 9th and 10th days and a blood infection on the 24th day The blood taken a week later still showed the parasites, and it also gave a positive Wassermann reaction   After continuing sick for a week more the animal recovered, failed to show parasites in the blood on later examination and remained well thereafter

In the case of the third patient there was severe inflammation of the bite wound but both the exuded serum and the blood were negative for *S minus* Animal inoculation was resorted to resulting in a positive blood infection on the 17th and a positive Wassermann reaction on the 19th day , but no evidence of any illness supervened

The fourth case was a woman , the healed scar in her broke out after 10 days but had healed again , blood was negative for the organism though tissue-juice from near the scar revealed its presence and, in addition, a few microfilaria She did not evince any pathological manifestation due to this latter infestation and her blood picture was that of a marked leucocytosis without any eosinophilia Her blood gave a strongly positive Wassermann reaction   The tissue-juice was injected subcutaneously into the right scrotum of a young guinea-pig and 2 c c of blood into the peritoneum of another from the same litter and both were caged together   The first one went down with the disease on the 69th day but the parasite was detectable for the first time only on the 97th day   Its Wassermann test at this time was positive (+)   The animal died on the 160th day   The one inoculated with blood remained well, thereby showing that in man a tissue infection without blood invasion or a filtration out of the parasites into the tissues subsequent to blood dissemination may occur

SUMMARY AND CONCLUSIONS

Four strains of *S minus* were studied with reference to their pathogenicity to guinea-pigs and in regard to certain immunological reactions they induce in the latter   Two of these were very pathogenic to guinea-pigs with a fatal termination

in one case on the 79th day and in the second on the 160th day   Another was definitely pathogenic but not fatal while the fourth was non-pathogenic   This difference in the pathogenicity, which may depend not only on the virulence of the parasite but also on the conditions in the guinea-pig host, probably explains the divergence in the results reported by many workers

Positive Wassermann reactions in the case of a large number of diseased guinea-pigs sufficiently prove that a complement-fixing antibody, which is identical with the syphilitic reagin in its physico-chemical properties, is produced in *S minus* infection in these experimental animals

Sub-passages in guinea-pigs tend to exalt the virulence of the parasite

The experimental disease in guinea-pigs proved to be readily amenable to Novarsenobillon

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A SHORT NOTE ON AN EPIZOOTIC AMONGST RABBITS DUE TO *SALMONELLA ÆRTRYCKE*

BY

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(*From the Central Research Institute, Kasauli*)

A FAIRLY severe epizootic amongst rabbits in the animal houses of the Central Research Institute, Kasauli, occurred in July with the start of the rainy season During the preceding 12 months the health of the animals was good and mortality low On the onset of the epizootic the breeding stock, normal and experimental animals, were affected alike On the whole the young and suckling rabbits suffered most Infection appeared to be species specific as regards the experimental animals Thus guinea-pigs kept in the same room were not affected

Symptoms were rather indefinite Nearly all infected animals went off their feed Marked diarrhœa was not a feature of the disease, but a few passed semi-solid fæces There were no paralytic symptoms In fact, but for one rather striking symptom—convulsive seizures just before death—there were very few symptoms at all There was no nasal or conjunctival discharge The outcome was fatal in 2 to 4 days

Post-mortem —With the exception of slight enlargement of the liver nothing abnormal could be seen in the viscera Spleen, brain, adrenals, intestine and kidneys appeared normal Lymph glands were not enlarged Lungs in a few cases showed bronchopneumonic patches

Cultural examination of the heart's blood and bile yielded a pure culture of a Gram-negative, actively motile bacillus having the biological characters of *Salmonella ærtrycke*

The vehicle of infection was undoubtedly food and drink probably infected through the agency of flies Several hundred white mice were kept in fly-proof cages in the same room as the infected rabbits but the stock remained healthy and uninfected

On the fourth day of the epidemic the remaining stock was vaccinated with an autogenous *ærtrycke* vaccine Partly because of this measure but mainly due to segregation and measures of fly destruction the epidemic ceased in about ten days

having killed 260 rabbits   It is interesting to note that rabbits used for the preparation of high titre sera for the *Salmonella* group of organism showed a low mortality rate as compared with those used for production of anti-cholera serum Of the thirty cholera rabbits only one survived, whereas out of forty-nine *Salmonella* rabbits fifteen survived

A SHORT NOTE ON VIABILITY OF BACTERIAL CULTURES

BY

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A NUMBER of stock laboratory cultures were tested for viability after having been kept in sealed tubes at room temperature for periods ranging from 12 to 19 years   It would appear from the results analysed below that this simple method of preservation of bacterial cultures is perhaps as good as any for maintenance of *Salmonella* and dysentery organisms   A few tubes of *P avisepticus*, *B pneumoniæ* (Friedlander) and *B proteus* X 19 were also tested and found to be alive after 12 to 13 years   Fourteen tubes of cholera and allied vibrios ranging in age from 12 to 18 years were tested but failed to yield any growth   *Streptococcus fœcalis* and *Staphylococcus albus* retained viability for 11 to 12 years   The medium used for preservation and sub-culture of *Salmonella* and dysentery organisms was mutton digest Douglas agar , for streptococci, staphylococci and *P avisepticus*, pigeon-blood agar

0 5 c c of sterile broth was added to each tube and the growth emulsified Sub-cultures were left in the incubator for 48 hours when the morphological, cultural, biochemical and serological characters of the strain were confirmed

Analysis of results —

Organism	Date of last sub culture	Number of tubes tested	RESULT
B typhosus	15–9–1914	2	Both alive
	29–9–1915	1	Alive
	15–4–1916	2	Both alive
	8–12–1920	1	Alive
	4–3–1921	8	7 alive, 1 dead

Organism		Date of last sub culture	Number of tubes tested	Result
B paratyphosus A	{	8–12–1920	1	Alive
		22–4–1921	4	2 alive, 2 dead
		30–12–1921	1	Alive
B paratyphosus B	{	15–4–1916	1	Alive
		8–12–1920	4	4 alive
B paratyphosus C		15–4–1921	3	Alive
B œrtrycke		8–12–1920	4	Alive
B enteritidis Gærtner		22–4–1921	5	Alive
B suipestifer		22–4–1921	5	Alive
B dysenteriœ Flexner Y		5–3–1921	5	4 alive, 1 dead
B dysenteriœ Flexner V		8–12–1920	4	4 alive
B dysenteriœ Flexner W		22–4–1921	2	1 alive, 1 dead
B dysenteriœ Flexner X		4–3–1921	2	2 alive
B dysenteriœ Flexner Z		22–4–1921	2	2 alive
B dysenteriœ Shiga		4–3–1921	1	Dead
B proteus X 19		22–4–1921	3	3 alive
B avisepticus		22–4–1921	2	1 alive, 1 dead
B pneumoniœ		22–4–1921	2	1 alive, 1 dead
Streptococcus fœcalis		19–7–1921	2	2 alive
Staphylococcus albus		4–3–1921	2	2 alive
Staphylococcus aureus		22–4–1921	4	4 dead

SPECIFICITY OF ANTIVENOMOUS SERA WITH SPECIAL REFERENCE TO SERA PREPARED WITH VENOMS OF INDIAN AND SOUTH AFRICAN SNAKES

BY

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THROUGH the courtesy of the Director, South African Institute of Medical Research, Johannesburg, I was supplied with the dried venom of Cape cobra (*Naia flava*) and a few phials of concentrated antivenene prepared against this and other venoms * The following experiments were carried out —

I   Toxicity of Cape cobra venom in omparison with that of Indian cobra venom,

II   Potency of Cape cobra antivenene against the venom of Indian cobra (*Naia naia*),

III   Potency of Indian cobra antivenene against the venom of Cape cobra (*Naia flava*),

IV   Potency of Indian cobra and Cape cobra antivenenes against the venom of banded krait (*Bungarus fasciatus*), and

V   Hæmolytic properties of venoms of Indian and Cape cobra as tested *in vitro*

Throughout these tests pigeons of 300 to 310 grammes weight were employed as experimental animals and all injections, whether of venom alone or venom and antivenene, were given intramuscularly into the pectoral muscles   The results were recorded after 18 to 20 hours

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* The antivenene is actually prepared against the venoms of Cape cobra and African vipers but for the purpose of the experiments to be described the antiviperine element may for convenience be ignored as having no bearing on the results   The Indian antivenene is likewise polyvalent and in it also only the anti cobra element is here considered

EXPERIMENT I   *Toxicity of Cape cobra venom in comparison with that of Indian cobra venom* —Venoms were accurately weighed up to four places of decimals and dissolved in normal saline to yield a solution of 1 mg per c c   From this stock solution further dilutions were made containing the desired amount of venom in 1 c e of saline   The following were the results obtained —

<center>TABLE I</center>

Snake venom	Dose, mg	RESULT	Snake venom	Dose, mg	RESULT
Naia flava venom	1	Pigeon died	*Naia naia* venom	1	Pigeon died
,,	0 8	,, ,,	,,	0 8	,, ,,
,,	0 7	,, ,,	,,	0 7	,, ,,
	0 6	,, ,	,	0 6	,
	0 5	, ,,	,,	0 5	,, ,,
	0 4	,, ,,	,,	0 4	,, ,,
,	0 3	,,	,,	0 3	,, survived
	0 2	,, survived	,,	0 2	,, ,
,	0 1	,, ,,	,,	0 1	,, ,,

From the above table it appears that 0 3 mg of *Naia flava* venom and 0 4 mg of *Naia naia* venom is lethal for a pigeon of 300 grammes weight   From a considerable personal experience in work with Indian cobra venom I find that the minimum lethal dose of this venom for a pigeon varies between 0 3 mg and 0 4 mg depending upon the thoroughness with which it is dried   Bearing this in mind I am of the opinion that the venoms of Cape cobra and Indian cobra possess about the same toxicity

EXPERIMENT II   *Potency of Cape cobra antivenene against the venom of Indian cobra* (Naia naia) —One c c of a solution of *Naia naia* venom containing 0 8 mg per c e was mixed with varying amounts of *Naia flava* antivenene and sufficient saline was added to bring the volume of the mixture to 2 c c   The tubes were

incubated at 37°C for 30 minutes before the venom-antivenene mixture was injected into pigeons   Table II gives the results —

TABLE II

Amount of *Naia flava* antivenene in c c	Amount of *Naia naia* venom in mg	RESULT
0 1	0 8	Pigeon died
0 2	0 8	,, ,,
0 3	0 8	,, survived
0 4	0 8	,, ,,
0 5	0 8	,, ,,

From the above experiment it appears that 0 3 c c of Cape cobra antivenene is sufficient to save a pigeon of 300 grammes weight against 0 8 mg of Indian cobra venom

EXPERIMENT III   *Potency of Indian cobra antivenene against the venom of Cape cobra* (Naia flava) —

TABLE III

Amount of *Naia naia* antivenene in c c	Amount of *Naia flava* venom in mg	RESULT
0 1	0 8	Pigeon died
0 2	0 8	, ,,
0 3	0 8	,, ,,
0 4	0 8	,, ,,
0 5	0 8	, survived
0 6	0 8	, ,
0 7	0 8	, ,

From Table III it appears that 0 5 c c of Indian cobra antivenene is sufficient to protect a pigeon of 300 grammes weight against 0 8 mg of Cape cobra venom

The results of experiments II and III clearly indicate that antisera prepared against the venoms of Indian cobra (*Naia naia*) and South African cobra (*Naia flava*) are mutually protective to a very marked degree

EXPERIMENT IV   *Potency of Indian cobra and Cape cobra antivenenes against the venom of banded krait* (Bungarus fasciatus) —The next point studied was the neutralizing effect of these sera on the venom of another colubrine snake, the banded krait (*Bungarus fasciatus*), which belongs to the same sub-family *Elapinæ* as the genus *Naia*

The minimum lethal dose of *B fasciatus* venom was found to be 3 mg for a pigeon of 300 grammes weight   The following table shows the results obtained with this venom .—

TABLE IV

Cape cobra antivenene (concentrated) in c c	B fasciatus venom in mg	RESULT	Indian cobra antivenene (concentrated) in c c	B fasciatus venom in mg	RESULT
0 1	3	Pigeon died	0 1	3	Pigeon died
0 5	3	,, ,,	0 5	3	,, ,,
1	3	,, ,,	1	3	,, ,,
2	3	,, ,,	2	3	,, ,,
4	3	,, ,,	4	3	,, ,,

It appears that neither Cape cobra antivenene nor the Indian cobra antivenene has any protective effect against the venom of banded krait and that *B fasciatus* venom is not even remotely related antigenically to the venoms of *Naia naia* or *Naia flava*

EXPERIMENT V   *Hæmolytic properties of venoms of Indian and Cape cobra as tested* in vitro —Red blood cells of various animals differ in their resistance to venom hæmolysis but for the same species of venom their susceptibility is more or less constant   For instance the venom of Indian cobra (*Naia naia*) will easily cause lysis of dog cells, while the venom of king cobra (*Naia bungarus*) will not hæmolyse dog cells even when large quantities of venom are employed (Lamb, 1905)   Seeing that the hæmolytic action of venom is so specific as to differentiate two such closely allied species of Indian cobras, this test of venom hæmolysis was used to find out if any differences existed in the hæmolytic elements of the venoms of Cape cobra and

Indian cobra   Red blood cells of dog, guinea-pig, rabbit, sheep, man, pigeon and horse were employed   One c c of a 3 per cent suspension of washed cells was added to 1 c c of various concentrations of venom   No complement or lecithin was added The results were recorded after 18 hours incubation at room temperature

TABLE V

One o c of 3 per cent sus pension of R B C of	Cape cobra venom in mg						Indian cobra venom in mg					
	1	0 1	0 01	0 001	0 0001	0 00001	1	0 1	0 01	0 001	0 0001	0 00001
Guinea pig	CH	CH	—	—	—	—	CH	CH	PH	—	—	—
Dog	CH	CH	—	—	—	—	CH	CH	PH	—	—	—
Sheep	—	—	—	—	—	—	—	—	—	—	—	—
Man	CH	CH	—	—	—	—	CH	CH	PH	—	—	—
Pigeon	PH	PH	—	—	—	—	PH	PH	—	—	—	—
Horse	tr	—	—	—	—	—	tr	—	—	—	—	—
Rabbit	PH	—	—	—	—	—	PH	—	—	—	—	—

Note —CH=complete hæmolysis   PH=partial hæmolysis
tr=trace hæmolysis   — = no hæmolysis

It appears that both these venoms exert the same hæmolytic effects on the erythrocytes of various species of animals   This shows that their hæmolytic principles are alike   Further proof of this assertion was given by the following experiment —

Experiment to ascertain if a serum prepared with Cape cobra venom has any neutralizing effect on the hæmolytic action of Indian cobra venom and vice versa — One c c of 3 per cent suspension of dog cells was added to each tube containing 1, 0 1, 0 01, 0 001, 0 0001 and 0 00001 mg of Cape cobra venom   0 2 c c of Indian cobra antivenene was then added and the tubes shaken and left for 18 hours at room temperature   To another similar set containing Indian cobra venom plus dog cells was added 0 2 c c of Cape cobra antivenene   The results were as follows —

With the exception of partial lysis in the tubes containing 1 mg of venom no lysis occurred in the rest of the tubes, thereby showing that Cape cobra antivenene could just as effectively inhibit Indian cobra venom hæmolysis as Indian cobra antivenene could inhibit Cape cobra venom hæmolysis

The antigenic principles must, therefore, be essentially the same to give rise to anti-bodies which are reciprocally protective and equally anti-hæmolytic against the two venoms   Taking into consideration toxicity, protection and *in vitro* experiments I am inclined to believe that both these cobras possess exactly the same type of neurotoxin and hæmolysin and that antivenene manufactured in India against *Naia naia* venom would be just as effective in cases of Cape cobra bites as if the antivenene had been raised against Cape cobra venom and vice versa

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A NOTE ON WILLIAMS' MODIFICATION OF EIJKMAN'S TEST AS APPLIED TO MADRAS WATERS

BY

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EIJKMAN's test has been found unreliable in this laboratory (Webster and Raghavachari, 1934) It was frequently negative when the sample of water under test could not be considered ' safe ' from a knowledge of local conditions and the results of other tests To confirm this finding, forty pure cultures of different strains of true *coli* recently isolated from water have since been tested in Eijkman's medium at 46°C Only 23 produced acid and gas within 48 hours

A modification of Eijkman's medium for which certain advantages are claimed has been reported from America (Williams *et al*, 1933) For 10 c c quantities of water they used 5 c c of a medium containing peptone (Witte) one per cent, beef extract 0 6 per cent and dextrose one per cent, with a pH of 6 6 Using 10 c c and 1 c c quantities of water they compared the new medium with standard methods They found that the new medium detected in 24 hours at 46°C all samples which were shown by standard methods to contain fæcal *coli* In fact the new medium showed up true *coli* in some samples which were passed by standard methods Not only so but the number of false positives was much less They claimed that with the lesser amount of carbohydrate in the new medium there was less acid production and all the true *coli* survived

Using MacConkey's bile-salt (1 5 per cent) lactose broth we are not seriously troubled with false positives in this laboratory In other words, when there is acid and gas in the 48-hour MacConkey broth culture we can generally demonstrate true *coli* after plating We, however, follow up six or ten colonies In a recent study in this connection (Raghavachari and Sitarama Iyer, 1934) it was found that of 338 lactose fermenters isolated from positive MacConkey broth cultures 68 7 per cent were true *coli* (M R +, indol+) Again of 67 samples giving a presumptive positive at 24 hours 65 yielded true *coli* (M R +) In over three-quarters of our samples the reading of the presumptive test is the same at 24 and 48 hours

A short series of comparative experiments was done with 10 c c quantities of water using (1) MacConkey broth at 37°C, (2) MacConkey broth at 46°C and (3) Williams' medium at 46°C The presence of acid and gas was noted after 24 hours

and the cultures, whether positive or negative, were plated out on MacConkey agar at the same time   If lactose fermenters appeared two were selected and identified by the usual tests—indol production, M R, V P, Koser's citrate and the four sugars saccharose, dulcite, adonite and inulin

TABLE

Comparative tests on forty samples of Madras waters (ten c c quantities only)

		coli only	coli and aerogenes	aerogenes only	No lactose fermenters
MacConkey broth at 37°C	Positive 34	15	7	12	
	Negative 6			1	5
MacConkey broth at 46°C	Positive 9	7			2
	Negative 31	9		1	21
Williams' medium at 46°C	Positive 2	1			1
	Negative 38	5	1	1	31

The results are summarized in the Table   Intermediaries have been grouped with *aerogenes* as there is no object in separating them in this particular case   The relatively large proportion of MacConkey broth cultures giving a positive presumptive at 37°C and failing to show true *coli* on confirmation is misleading as only two colonies were followed up instead of the six or ten we use in routine work   Only two positive presumptives in Williams' medium at 46°C may be compared with 22 confirmed tests with MacConkey broth at 37°C   Some negative tubes of Williams' medium, however, yielded true *coli* on plating   It appears that *aerogenes* and intermediaries are inhibited almost entirely in Williams' medium at 46°C but that *coli* also often fails to thrive

The results with MacConkey broth at 46°C were very irregular   The *aerogenes* and intermediaries were certainly almost completely eliminated but in no less than twelve instances samples showing true *coli* at 37°C gave completely negative results at 46°C   In five cases confirmation gave true *coli* from the tubes at 46°C and only *aerogenes* from the tubes at 37°C.   This is explained by the inhibition of *aerogenes* in the former and the inadequate number of colonies followed up from the latter   Once *neopolitanus* was recovered at 46°C when the presumptive test at 37°C was negative

Streptococci were sought for in all these cultures   They were not detected in either of the sets of MacConkey broth cultures but five examples of heat resistant streptococci were recovered from the cultures in Williams' medium at 46°C

The samples examined were chiefly well waters from the Madras Presidency but included examples of river, gallery, impounded reservoir and bore-hole waters

SUMMARY

MacConkey bile-salt lactose broth has for many years been found satisfactory for the presumptive *coli* test on Madras waters and presumptive positives can generally be confirmed   Williams' modification of Eijkman's test is found to be much less sensitive for the presumptive test   Although it largely eliminates *aerogenes* and intermediaries yet it frequently fails to demonstrate true *coli*

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STREPTOCOCCUS FÆCALIS IN MADRAS WATERS

BY

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As evidence of the pollution of water from the mammalian intestine the 'microbes of indication' are *Bacterium coli* and its allies and the streptococcus (Eyre, 1930) Dihle (1921) concluded from his detailed study of intestinal streptococci that they may be divided into two groups The true enterococcus or *Streptococcus fæcalis* is heat-resistant, surviving 60°C for half an hour, and often ferments mannite The non-heat-resistant streptococcus is probably usually a surviving salivary form and often attacks raffinose

Savage and Reed (1917), working in England, found streptococci to be present in about half of 443 samples of water when quantities of 0 1 c c or 1 0 c c were tested Leiter (1929) on the other hand, working in America, reported cocci to be rare in water-supplies Minett (1930) failed to find streptococci in the various raw waters in Hong Kong As a result of the work of Clemesha (1912) the presence of streptococci in tropical waters is regarded as evidence of very recent pollution He found that although they can be demonstrated in very small quantities of mammalian fæces (0 00001 g) yet they are extremely rare in tropical water-supplies He also considered that streptococci die out rapidly in stored water and that sunlight is not the important lethal factor It may be noted that Clemesha's fæcal streptococci did not ferment mannite Houston (1932) described a technique for the isolation of heat-resistant streptococci from the positive cultures in MacConkey bile-salt broth used in the presumptive test for *coli* in water He noted that bile-salt is somewhat inhibitory to streptococci Other writers consider that bile-salt inhibits some streptococci but not *Str fæcalis* (Thresh *et al* , 1933)

In this laboratory attention was directed to water streptococci by the unexpected results found with Eijkman's test (Webster and Raghavachari, 1934) We examined 60 samples of water by culture in Eijkman's medium at 46°C followed by sub-culture on both MacConkey agar and on a plain lactose agar Streptococcus-like colonies were frequently detected on the plates of both media and in 16 cases these proved to be streptococci, capable of surviving half an hour at 60°C Only

twice were these recovered from both plates relating to the same sample of water so they were apparently frequently missed   Their reactions were as follows —

	Lactose litmus	Mannite	Raffinose	Reduction of nitrite
From lactose agar				
4	A	0	0	0
1	A	0	A	0
From MacConkey agar				
6	A	0	0	0
1	A	0	A	0
2	0	0	A	0
2	0	0	0	0

Later a series of 40 tests with 10 c c quantities of water in Williams' modification of Eijkman's medium (Williams *et al*, 1933) yielded five examples of heat-resistant streptococci   These failed to ferment either mannite or raffinose

Fifty-five MacConkey broth tubes giving a positive presumptive *coli* test with 20 c c quantities of water after 24 hours' incubation at 37°C and selected at random from samples received for routine analysis were then tested for heat-resistant streptococci after Houston's procedure   One c c was added to 9 c c sterile water, avoiding side smearing, and this was heated to 60°C for half an hour   No streptococci were detected in sub-cultures of these on agar

It was then thought that perhaps streptococci missed by this method might be shown up by some form of secondary enrichment   A number of 20 c c samples of water in MacConkey broth was, therefore, tested after 24, 48, 72 and 96 hours at 37°C   One c c was added to 9 c c sterile water and heated to 60°C for half an hour as before   Instead of plating this out at once, however, the mixture was added to MacConkey broth and glucose broth respectively, 5 c c to each, and after 24 hours at 37°C these cultures were plated out on MacConkey agar   The results are shown in the Table.

TABLE

Modified Houston's test for fæcal streptococci in Madras waters

(Twenty c c samples, after enrichment in MacConkev broth tested by heat and secondary enrichment)

(*a*) *Secondary enrichment in MacConkey broth*

PRIMARY ENRICHMENT	24 HOURS		48 HOURS		72 HOURS		96 HOURS	
Coli test	Number	Streps	Number	Streps	Number	Streps	Number	Streps
Presumptive positive	79	1	69	1	52		17	
Presumptive negative	70		64		36		12	
TOTALS	149	1	133	1	88		29	

(*b*) *Secondary enrichment in glucose broth*

PRIMARY ENRICHMENT	24 HOURS		48 HOURS		72 HOURS		96 HOURS	
Coli test	Number	Streps	Number	Streps	Number	Streps	Number	Streps
Presumptive positive	45	1	43	4	32	1	15	
Presumptive negative	26	1	33		14	2	8	
TOTALS	71	2	76	4	46	3	23	

Streptococci were certainly recovered occasionally but it is certain that they were more often missed Only once were they detected in the same sample after

both forms of secondary enrichment and only once did they appear in more than one of the tests on the same sample with the same enrichment procedure   Most of the samples were tested on three occasions   Glucose broth was the more successful medium for secondary enrichment   Most of the sub-cultures from MacConkey broth were sterile but, as might be expected, sub-culture from glucose broth often showed up Gram-positive spore-bearers   Streptococci when detected were usually in pure culture and showed as tiny bright red or pale pink colonies on MacConkey agar

The numbers are too small to decide which period of primary incubation was most successful but streptococci were actually recovered at the 24-, 48- and 72-hour periods   In three cases a sample positive for streptococci had given a negative presumptive for *coli*   The strains isolated were all mannite negative but four produced acid in raffinose   It was noticed that at least five of the strains had attacked mannite after 72 hours   The various samples showing streptococci included examples from all the usual sources of water-supply—rivers, wells, tube-wells, galleries and impounded reservoirs—and there was one positive in a sand-filtered water

Some experiments on the longevity of these streptococci in stored water were commenced   The cultures were recently isolated from water   The 24-hour growth from half an agar slope was added to a Winchester quart bottleful of sterilized water and this was stored in the laboratory in diffused daylight at a temperature usually between 85°F and 95°F   Samples were taken every few days   The longest periods after which the streptococci were recovered were 16 days in the case of distilled water and 20 days in the case of river-water

SUMMARY

Streptococci with the heat-resistant character of *Str fæcalis* are not at all uncommon in Madras waters   They have been demonstrated in 10 c c or 20 c c quantities in 19 samples of water out of 100, in glucose broth cultures at 46°C   They are regularly mannite negative but sometimes attack raffinose   Some strains ferment mannite later than the orthodox 48 hours   These streptococci may survive in stored water for at least 20 days

They are not detected in the course of the routine bacteriological examination of water and have not been recovered from cultures in MacConkey broth by Houston's procedure   By a modification of Houston's method, introducing secondary enrichment after heating, an occasional sample could be proved to contain fæcal streptococci   A reliable method of demonstrating these streptococci when present has not, however, been devised   The most promising procedure unfortunately requires incubation at 46°C —a serious drawback for routine work

The author, who has had to stop this work owing to transfer, has written this preliminary report to draw attention to the possibility of useful information being obtained by further study of fæcal streptococci in tropical waters   At present the bacteriological evidence of the fæcal pollution of water is almost limited to the demonstration of *coli*   Some test of a confirmatory nature, simple enough for adoption as a routine, would be welcomed by all water bacteriologists

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BLOOD GROUPS AND HEREDITY

BY

CAPTAIN P N MITRA, M B , D T M

THE object of the present short note is to bring forward some evidence of the hereditary transmission of blood groups   The materials dealt with by me relate to two thousand individuals at Dibrugarh, Assam, and include both healthy and sick   The latter were consecutive cases reporting sick at a hospital as out-patients and admitted cases   The former comprised chiefly medical students   On the whole the population dealt with might be taken as a fair sample of the population of upper Assam although many of the students will have come from other areas

Grouping technique applied

The slide technique was used   A glass slide was marked into three areas with a grease pencil   A drop of group A serum, group B serum and normal saline was put on each area   Two drops of a 5 per cent suspension of red blood cells in normal saline were then added to each drop and the contents intimately mixed   The result was read with the naked eye and doubtful results were checked by the microscope

In the event of a very feeble reaction the result was checked by another serum or two of the same group   Whether the blood groups are transmitted hereditarily or not could be proved in two ways, directly or indirectly—

(1) By the direct method one would have to work out the blood groups of both parents and children   This method there has been no opportunity of applying to the present series

(2) The indirect method is based on the assumption that if in a community the number of persons of groups A and B are known then the number of persons of group AB, which has arisen out of a combination of groups A and B, can be calculated

Von Dungern and Hirsfeld (1910) who studied this question concluded that the blood-group component is transmitted through two independent pairs of chromosomes and they published a formula for calculating the probable number of persons of group AB   On the other hand Bernstein (1925) thought that the hypothesis of three allelomorphs, viz , A, B and O, combining with each other two by two and

located in the same part of the chromosome agrees more closely with the facts He also published a formula for finding the number of persons of group AB Both these indirect methods have been applied in dealing with the figures collected for this inquiry These figures are given below —

Blood group	Number in blood groups	Percentage of total
A	491	24 55
B	651	32 55
AB	185	9 25
O	673	33 65
TOTAL	2,000	

On Von Dungern's hypothesis, as modified by his pupils Halbar and Mydlarski (1923)

let $p =$ frequency of A (agglutinogen)

$\quad q = \qquad$,,   ,, a (agglutinin)

$\quad r = \qquad$,,   ,, B

$\quad s = \qquad$,,   ,, b

Then $p + q = 1$ or $(p + q)^2 = 1$ and $r + s = 1$ or $(r + s)^2 = 1$

or $(p + q)^2 \times (r + s)^2 = 1$ or $(p^2 + 2pq + q^2)(r^2 + 2rs + s^2) = 1$

or $p^2 r^2 + 2p^2 rs + p^2 s^2 + 2pqr^2 + 4pqrs + 2pqs^2 + q^2 r^2 + 2q^2 rs$

$+ q^2 s^2 = 1$

$\quad$ Group $\ O = q^2 s^2$

$\qquad$,, $\quad A = p^2 s^2 + 2pqs^2 = s^2 (p^2 + 2pq) = s^2 \ (1{-}q^2)$

$\qquad$,, $\quad B = q^2 r^2 + 2q^2 rs = q^2 (r^2 + 2rs) = q^2 \ (1{-}s^2)$

$\qquad$,, $\quad AB = p^2 r^2 + 2pqr^2 + 2p^2 rs + 4pqrs = (1{-}q^2)(1{-}s^2)$

From the first three above s^2 and q^2 are calculated

$$s^2 = \frac{O}{O + B} \text{ and } q^2 = \frac{O}{O + A}$$

$$AB = \left(1 - \frac{O}{O+A}\right)\left(1 - \frac{O}{O+B}\right)$$

$$= \left(1 - \frac{33\ 65}{33\ 65 + 24\ 55}\right)\left(1 - \frac{33\ 65}{33\ 65 + 32\ 55}\right)$$

$$= \left(1 - \frac{33\ 65}{58\ 2}\right)\left(1 - \frac{33\ 65}{66\ 2}\right) = \frac{24\ 55}{58\ 2} \times \frac{32\ 55}{66\ 2}$$

$$= 0\ 20 \text{ or } 20 \text{ per cent}$$

This is *much above* the *observed percentage which is* 9 25 only

According to Bernstein's theory the calculation will be as follows —

Let $p = $ frequency of A

$q = $,,   ,, B

$r = $,,   ,, O

Then $p + q + r = 1$ or $(p + q + r)^2 = 1$ or $p^2 + q^2 + r^2 + 2pq + 2pr + 2qr = 1$

- Group $O = r^2$
  - ,, $A = p^2 + 2pr$
  - ,, $B = q^2 + 2qr$
  - ,, $AB = 2pq$

Group O + Group $A = r^2 + p^2 + 2pr = (r + p)^2$ or $r + p = \sqrt{Group\ O + Group\ A}$

or $p = \sqrt{Group\ O + Group\ A} - r = \sqrt{Group\ O + Group\ A} - (1-p-q)$

or $q = 1 - \sqrt{Group\ O + Group\ A}$

Similarly $p = 1 - \sqrt{Group\ O + Group\ B}$ and $r = \sqrt{Group\ O}$

So $Group\ AB = 2pq = 2 \times (1 - \sqrt{Group\ O + Group\ B})$ $(1 - \sqrt{Group\ O + Group\ A})$

$= 2 \times (1 - \sqrt{0\ 3365 + 0\ 3255})\ (1 - \sqrt{0\ 3365 + 0\ 2455})$

$= 2 \times (1 - \sqrt{0\ 6620})\ (1 - \sqrt{0\ 5820}) = 2 \times (1 - 0\ 81)\ (1 - 0\ 76)$

$= 2 \times 0\ 19 \times 0\ 24$

$= 0\ 0912$ or $9\ 12$ per cent

The observed percentage being 9 25, the agreement is very close

Conclusion

The human blood group factor, as observed at Dibrugarh, is transmitted by heredity, according to the triple allelomorph theory of Bernstein

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SPECTROGRAPHIC EXAMINATION OF SOME INDIAN MILKS

BY

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(*From the Nutı ıtıon Research Laboratorıes, I R F A , Coonoor*)

THE spectrographıc method of analysıng bıologıcal materıals ıs regularly employed in thıs laboratory both as a routıne and for research purposcs   Mılk ıs among the varıous food-stuffs recently analysed ın thıs way   The present paper records the results obtaıned wıth human mılk and wıth that of the cow, goat and buffalo

The analysıs consısted ın —

    (*a*) Examınatıon for mıneral elements that are not detectable by ordınary chemıcal methods

    (*b*) Estımatıon of the vıtamın A content of the mılk

The detectıon of the elements was done by the method of emıssıon spectra, whıle the estımatıon of vıtamın A was made by the method of absorptıon of lıght waves ın the ultra-vıolet regıon of the spectrum

PART I.

Emıssıon Spectra

Materıal —The materıals used ın thıs study consısted of five samples of each varıety of mılk, vız , of cow, goat, buffalo and woman   Ten to twenty-five cubıc centımetres of fresh mılk were drıed slowly ın a sılıca crucıble at a low temperature over a small Bunsen flame   Spccıal care was taken to avoıd any contamınatıon wıth foreıgn materıals   The drıed contents were then heated ın an electrıc muffle furnace and converted ınto ash   Quantıtatıve estımate of the ash content of the

(499)

different varieties of milk was made for two samples of each with the following results —

TABLE I

Variety of milk	Quantity of milk ashed in c c	Percentage of ash in sample I	Percentage of ash in sample II	Mean percentage of ash
(1) Human milk	10	0 30	0 28	0 29
(2) Cow ,,	25	0 60	0 74	0 67
(3) Buffalo ,,	25	0 72	0 66	0 69
(4) Goat ,,	25	0 80	0 85	0 825

The above findings agree with those of Heubner, Heineman and others quoted by Hutchison (1916), Morse (1927) and Clayton (1932)   The ashes were finely powdered in an agate mortar and stored carefully until subjected to spectrographic analysis

Technique.

The technique of obtaining emission spectra of the ashes was essentially that described in a previous paper (Boyd and De, 1933)   Twenty-five mg of each ash (only 10 mg in the case of human milk ash) were transferred to a suitably prepared H S brand graphite electrode and the arc struck, using a current of 6 amperes at 220 volts D C   The spectrograph used was Hilger's all-metal medium-sized quartz spectrograph, E316, with internal wave-length scale   In juxtaposition to each spectrum a photograph of the internal wave-length scale was taken to enable the spectral lines to be read conveniently   To ensure the accuracy of such readings, a spectrogram of R U powder was also taken on each plate, thus enabling the position of unknown spectral lines to be detected accurately   To guard against any impurities in the electrodes, a blank spectrogram was taken on each plate   The spectrograms were searched for 'Raies Ultimes' of the elements listed in Table II   Though the elements were detected qualitatively a rough quantitative comparison had been made for certain elements by spectrographing equivalent amounts of different milk ashes   The figures in Table I, and those of Heubner and Heineman, show that the percentage of ash in human milk is low, being only from one-third to one-half of that in other milks   Hence to compare roughly the analytical results 10 mg of the human milk ash was spectrographed, while 25 mg were taken in other cases

Discussion and Results

Blumberg and Rask (1933) have made a similar analysis and quote spectrographic findings by Wright and Papish (1929) and Zbinden (1931) for a number of specimens of cow milk from the United States of America, Great Britain and widely distributed European sources   The elements, calcium, magnesium, sodium, potassium and phosphorus, were found in large quantities in all the milk samples examined   The complete list of elements, detected by these investigators

include Al, Ba, Cr, Cu, Fe, Pb, Li, Mn, Rb, Si, Sr, Sn, Ti, V and Zn Ag was also detected in some human milks Their results agree with respect to a few elements and differ widely in regard to the presence or absence of certain other elements The purpose of the present communication is to record a new set of data (qualitative) on the biologically rare elements present in different Indian milks

These data are as follows —

(1) The elements Na, Ca, Mg, P, K, Cu, Fe, Al, B, Mn, Pb, Ti, V and Zn were detected in all milk samples examined

(2) Ba, Co, Ni and Sn were absent in all, and the presence of Cr, Li, Rb and Sr was doubtful

(3) Ag—the presence of silver was doubtful in two samples of human milk

(4) Si—this element was present in cow milk in large amounts, while its presence was doubtful in other cases

(5) P—this element was the least plentiful in human milk (Morse, *loc cit*)

(6) Ca—was most abundant in buffalo milk, least so in human milk (Morse, *loc cit*)

(7) Cu and Fe—maximum in human milk—minimum in buffalo and cow milk (Wallgren, 1933)

Table II shows how the above qualitative results compare with those of previous workers, omitting Ca, Mg, P, K and Na concerning the presence of which in milk there is no question

TABLE II

Symbols	Wright and Papish	Zbinden	Blumberg and Rask	Drea	De
Al	+	+	*ι*	+	+
Ba	—	0	+	+	—
B	+	0	+	+	+
Cr	—	+	—	+	?
Cu	+	+	+	+	+
Fe	+	+	+	—	+
Pb	—	+	—	+	+
Li	+	0	+	+	?
Mn	+	+	?	—	+
Rb	+	0	+	+	?
Si	+	—	?	?	+
Sr	+	0	+	+	?
Sn	—	+	—	+	—
Ti	+	+	+	+	+
V	+	+	?	+	+
Zn	+	+	+	+	+

+ = present , — = absent, 0 = not tested for , ? = doubtful

To the above list of elements Drea (1934) adds silver and molybdenum   These he detected only in milk of cows drinking water and eating alfalfa containing strong traces of silver and molybdenum

PART II.

Estimation of Vitamin A Values.

This part of the present communication records the results of experiments on the quantitative determinations of vitamin A of different varieties of milk by the spectrophotometric method   The constant presence of a band at 3,280 A U in the absorption spectra of substances which restore the normal growth of rats deprived of vitamin A, has suggested to many authors the possibility of measuring the quantity of vitamin A present by the intensity of this absorption band   Morton *et al* (1931), comparing the values of biological tests for vitamin A with spectrographic and colorimetric determinations, found satisfactory agreement between the biological and physical tests   Chevallier and Chabre (1933), working with fish oils, report that if the pigments and free acid contents of the materials be low or be previously removed by alcoholic extraction the intensity of the band at 3,280 A U permits very accurate measurement of the vitamin A

Macwalter *et al* (1934) prefer to use the unsaponifiable fraction for such estimations

Materials and Technique.

The vitamin A values have been estimated in each of five samples of cow, goat, buffalo and human milk   Twenty c c to sixty c c of milk were allowed to soak into a sheet of blotting paper , the sheet was then dried and the fat extracted with ether in a Soxhlet apparatus   Three different procedures were tried to prepare the materials for spectrophotometric study   These were   (1) to examine the fats directly (in ether solutions—of suitable strength), (2) to examine the same after several alcoholic washes and (3) to examine the ether extract of the unsaponifiable fraction

The first two procedures had to be rejected as the materials obtained by these methods showed too much irrelevant absorption at 3,280 A U and in the shorter-ultra-violet region   The last named procedure was found satisfactory and has been used in the present investigation

The fat was saponified under reflux for about 45 minutes with 15 c c to 25 c c. of 10 per cent alcoholic potash   Fifty c c of water were added and the mixture was cooled to 0°C in ice   About 50 c c of ether were then added, followed by an addition of 100 c c cold water   The mixture (in a separating funnel) was thoroughly shaken and, when ether separated out in about 3 to 5 minutes, it was drawn off and the aqueous alcoholic potash was then extracted three successive times with additional portions of ether of 30 c c each   The combined ether solutions were thoroughly washed with water repeatedly and then concentrated to the required volume by means of an electric filament lamp heater   Absorption curves have been prepared for all and some are shown here (Figs 1 to 7) for illustrative purposes
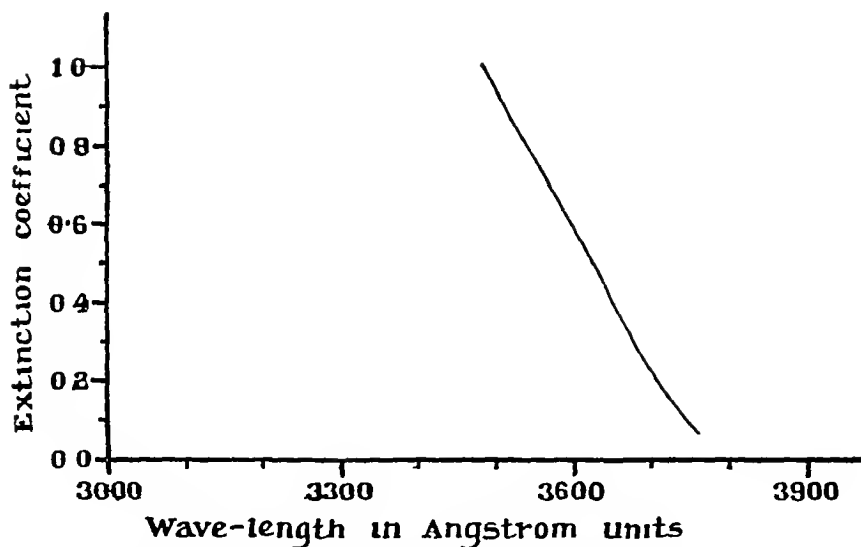
Fig 1   Absorption curve of cow milk fat, spectrographed directly   The fat from 30 c c of fiesh milk was dissolved in 50 c c ether
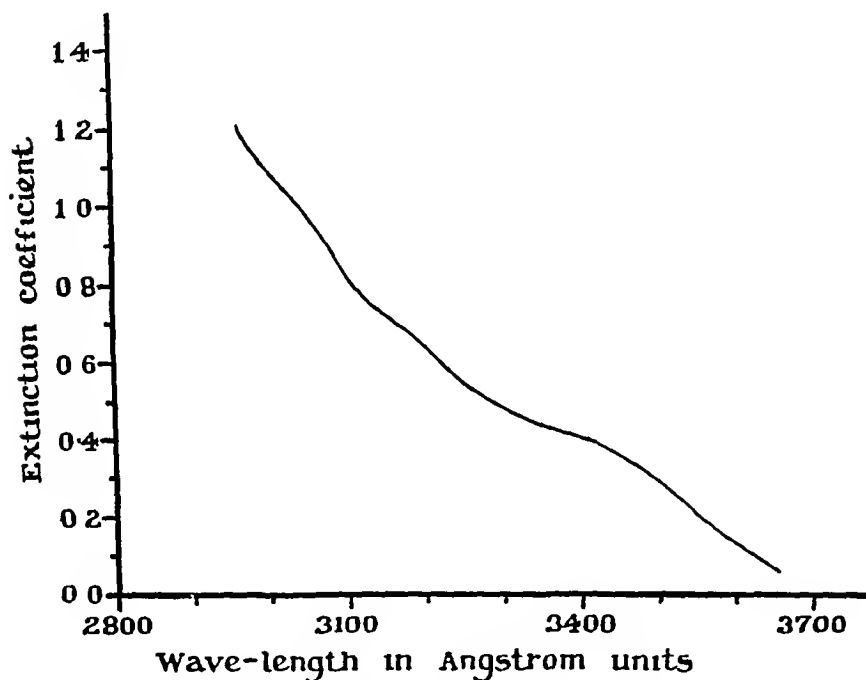

Fig 2   Absorption curve of cow milk fat   The fat from 25 c c of fresh milk was repeatedly washed with alcohol, and dissolved in 50 c c ether
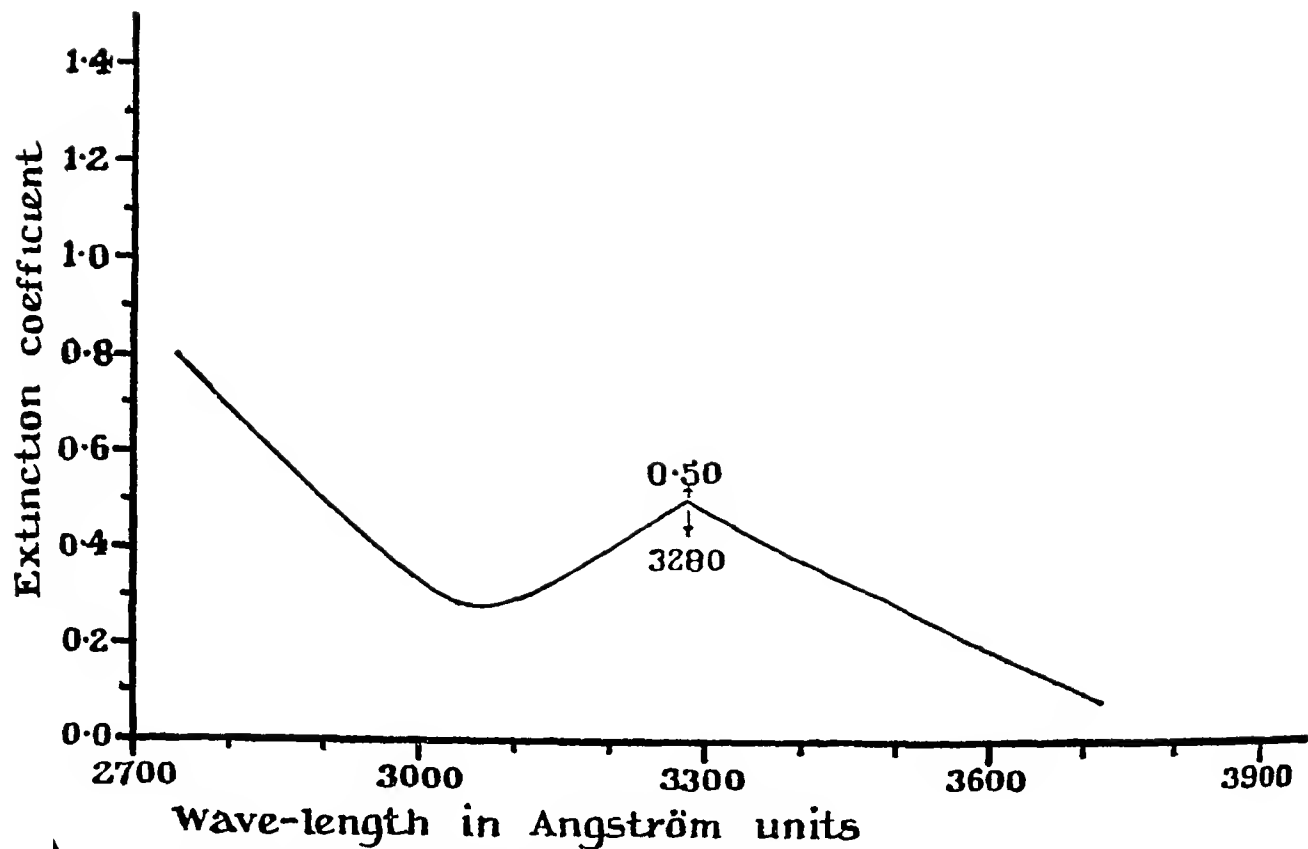
Fig 3   Absorption curve of cow milk fat   The unsaponifiable fraction of the fat from 30 c c fresh milk was
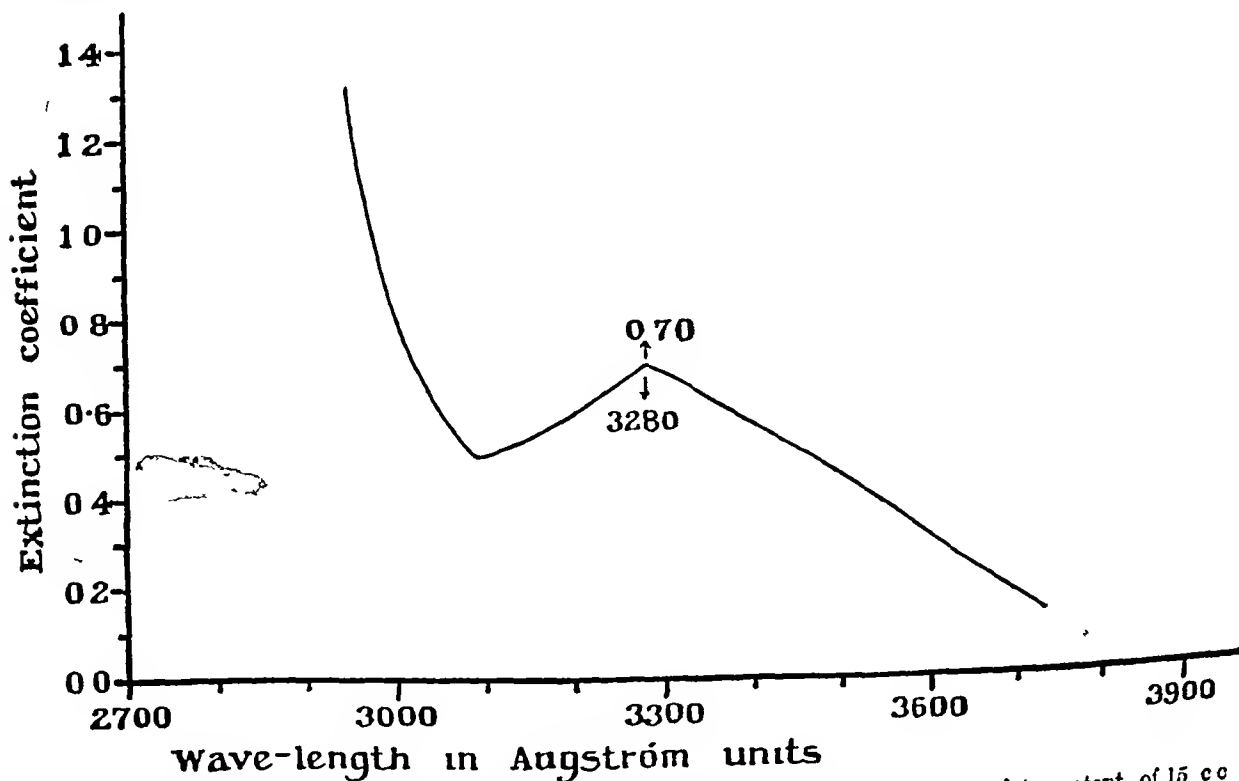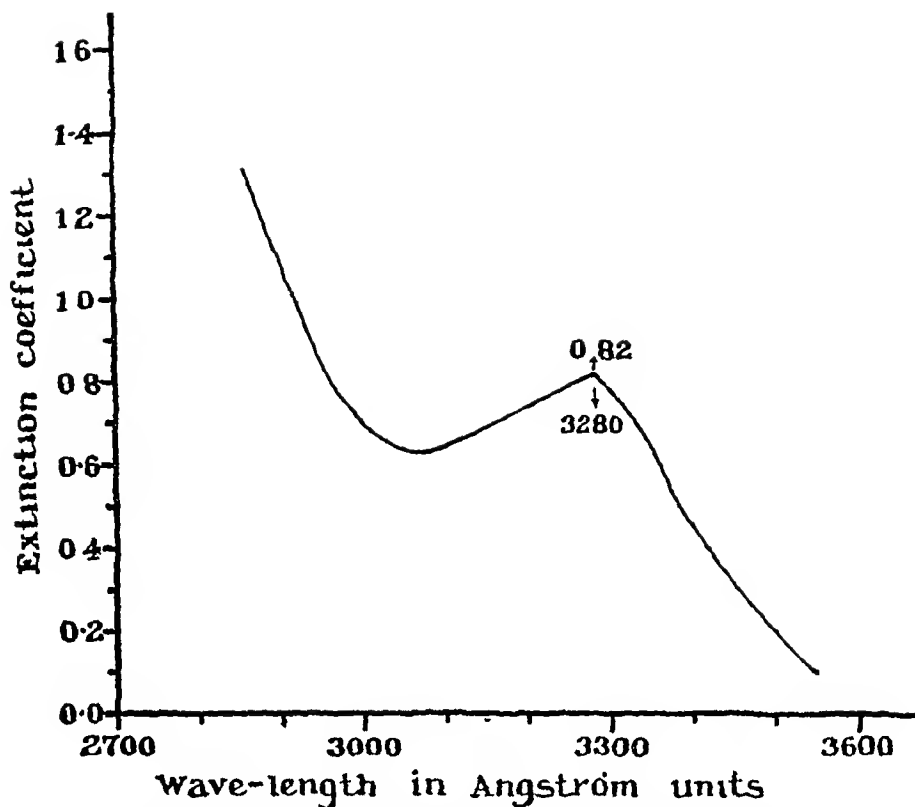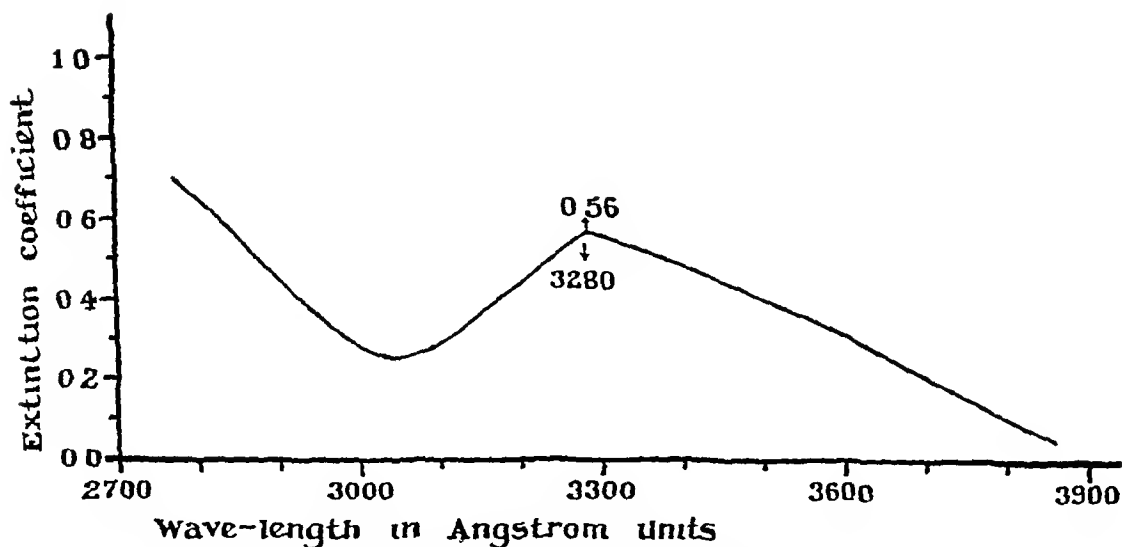·acted with ether and the ether solution was concentrated to 5 c c

Fig 4   Absorption curve of human milk fat   The unsaponifiable fraction of the fat content of 15 c c
fresh milk was extracted with ether and the solution concentrated to 2 5 c c

Fig 5   Absorption curve of goat milk fat   The unsaponifiable fraction of the fat from 30 c c   fresh milk was extracted with ether and the final solution concentrated to 5 c c

Fig 6   Absorption curve of buffalo milk fat   The unsaponifiable fraction of the fat from 50 o c   fresh milk was extracted with ether and the solution concentrated to 10 c c

Absorption pictures were taken with Hilger's medium-sized quartz spectrograph, E316, equipped with the rotating sector   The source of light was a spark (4 mm gap) between steel-tungsten electrodes   The voltage was 15,000 and the initial exposure 10 seconds   The liquid containers were 1 cm quartz cells

Absorption curves have been prepared for a sample of cod-liver oil   Vitamin A content of milk has been compared to it   Drummond *et al* (1932) isolated a pure sample of vitamin A and found that one physiological rat dose lies between 0 1 γ and 0 5 γ of their preparations   Carr and Jewell (1933) have been able to
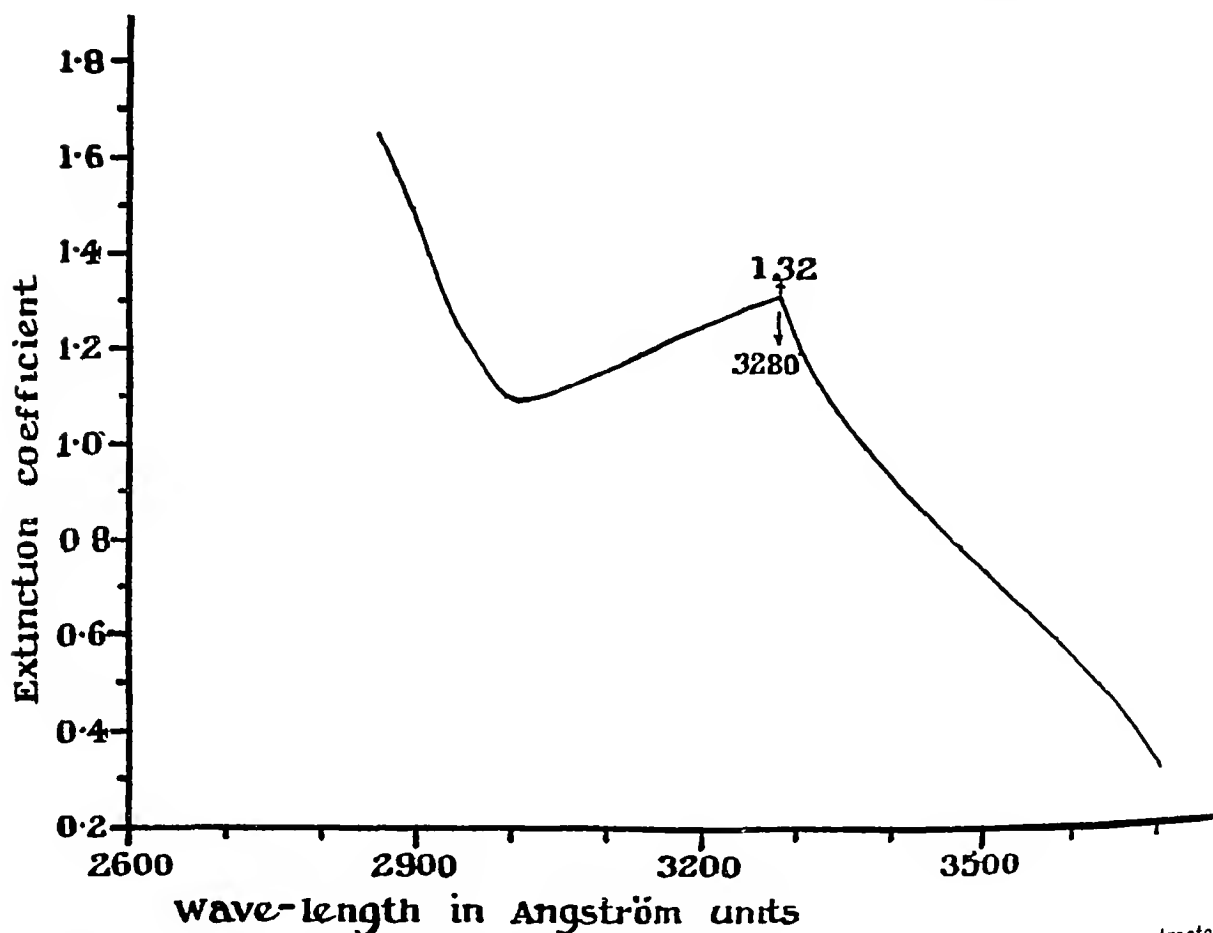
Fig 7 "Absorption curve of 2 per cent solution of cod liver oil   The unsaponifiable fraction was extracted with ether

isolate a purer sample which is the purest preparation of the vitamin so far known   It showed an absorption band at 3,280 Å U (Carr and Jewell, *loc cit*), the extinction coefficient for one per cent solution in 1 cm cell being 1,600   This figure of Carr and Jewell has been adopted here as a standard of comparison, for calculations of the vitamin A content of milk in absolute units   Assuming 0 3 γ (mean of 0 1 γ and 0 5 γ, Drummond *et al*, *loc cit*) as an average rat dose, vitamin A content of milk has also been expressed in U S P rat units

The data set out in the last three columns of Table III show that cow, goat, human and buffalo milk contain similar amounts of vitamin A   Luck (1933)

TABLE III

Variety of milk		Quantity of milk from which fat was extracted (in c c)	Volume of the final concentrated ether extract of the unsaponifiable fraction (in c c)	Observed extinction coefficient at wave length 3,280 A U	Vitamin A content per 100 c c of milk in absolute units (in γ)	Mean vitamin A content per 100 c c of milk in absolute units (in γ)	Mean vitamin A content per 100 c c of milk (in rat units)	Mean vitamin A content per 100 c c of milk expressed in equivalent weight of cod liver oil (in g)
Human milk	1	15	2 5	0 70	72 92			
	2	25	10 0	0 42	105 00			
	3	15	5 0	0 34	70 83	81 00	270 00	0 2025
	4	15	2 5	0 60	62 50			
	5	15	2 5	0 90	93 75			
Cow milk	1	30	5 0	0 50	52 08			
	2	60	20 0	0 41	85 42			
	3	30	10 0	0 40	83 33	70 12	233 70	0 1753
	4	50	10 0	0 54	67 50			
	5	60	15 0	0 40	62 50			
Goat milk	1	30	5 0	0 82	85 42			
	2	30	10 0	0 36	75 00			
	3	50	10 0	0 70	87 50	70 94	236 5	0 1773
	4	30	5 0	0 50	52 08			
	5	60	7 5	0 70	54 69			
Buffalo milk	1	50	10 0	0 56	70 00			
	2	30	5 0	0 60	62 50			
	3	40	5 0	0 70	54 69	63 17	210 6	0 1579
	4	60	8 0	0 70	58 33			
	5	40	5 0	0 90	70 31			

reports results of biological tests by Faberi, Sandicchi, MacLeod and others, that vitamin A activities of ewe, cow, ass and human milk are similar and lie between 1 3 to 2 rat units per gramme

Discussion and Summary.

The results of the present investigation agree with available biological data reported by previous workers   The spectrographic method of determining the vitamin A content of milk is very useful , it is much quicker than biological tests and gives equally reliable results provided sufficient care be taken to remove pigments, fatty acids and other irrelevant substances from the materials under test

The present observations were restricted to human, cow, goat and buffalo milk, since these are the only milks in common use in India   No consideration has, however, been made regarding the dependence of vitamin A and the mineral constituents of milk on season, locality and diet   According to Macy *et al* (1927), Luce (1924) and Chick *et al* (1926) the qualities of milk do depend on such factors Hence the result of the present investigation represents only the average composition of milk   The results observed can be summarized as follows —

(1) Human, cow, goat and buffalo milk contain similar quantities of vitamin A   Vitamin A content per 100 c c of each variety of milk lies between 50γ and 105γ (average 71 3γ or 238 rat units) and is equivalent to 0 15 g to 0 2 g of cod-liver oil

(2) Human milk is richer in copper and iron than the other milks examined, but it is very poor in phosphorus and calcium

(3) Cow milk is richer in calcium and poorer in Fe and Cu   The element silicon is plentiful in cow milk but its presence is doubtful in other milks

(4) Buffalo milk is poorer in iron, copper and manganese, but richer in calcium, magnesium and sodium

(5) Goat milk, unlike milk from other species, contains all the elements concerned in fair amounts

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A SPECTROGRAPHIC STUDY OF THE VITAMIN A CONTENT OF SOME OILS AND FATS

BY

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THE present paper records the results of experiments on the quantitative estimation of the vitamin A content of some common oils and fats   According to Morton *et al* (1931), Chevalier and Chabre (1933), Macwalter *et al* (1934) and others, vitamin A has a specific absorption band at 3,280 A U , and if sufficient care be taken to remove pigments, fatty acids and other interfering substances from the materials under test, the absorption band at 3,280 A U permits of very accurate estimation of their vitamin A values Careful spectrographic observations gave results in close agreement with those obtained by biological tests   Crews and Cox (1934), Lindholm (1934), Bowden and Snow (1932), Edisbury *et al* (1933) and numerous other workers established the specificity of this band for vitamin A and also advocated the use of such methods for vitamin A assay   Since pure vitamin A was not available for purposes of comparison, the results reported so far were only comparative Drummond *et al* (1932) reported that they had isolated pure vitamin A which gave $E \frac{1 \text{ per cent}}{1 \text{ cm}} = 1,350$ Since then experiments have been done by others (Baumann and Steenbock, 1933), to express the vitamin content of different materials in absolute units   Recently Carr and Jewell (1933) claimed to have isolated a purer concentration of vitamin A which gave $E \frac{1 \text{ per cent}}{1 \text{ cm}} = 1,600$ Accepting this value as a standard of comparison, the vitamin A contents of some oils and fats have been determined and the results of these determinations are reported in the present communication   Baumann and Steenbock (*loc cit*) quoted results of biological tests by Euler and Karrer (1931) and Drummond *et al* (*loc cit*), according to which one physiological rat dose of pure vitamin A lies between 0 1γ to 0 5γ   Taking 0 3γ (average of 0 1γ and 0 5γ) as the basis of calculation the vitamin A content of the various oils and fats examined has been expressed in rat units

Technique.

Details of the method of absorption spectrography and of preparing materials for photometric use arc essentially those described previously (Sankaran and De, 1934 , De, 1935)   One to ten grammes of the materials were saponified with 10 per cent alcoholic potash and the unsaponifiable fractions extracted with ether A solution of suitable strength of this extract was then spectrographed   In a few cases (butter, ghee, mustard oil, shark oil) the ether extracts were found to be coloured , these were slightly acidified with a few drops of dilute hydrochloric acid and filtered through charcoal   The filtrate obtained was colourless and has been used for the spectrographic study   In addition to examinations of the unsaponifiable fractions as stated above, some independent experiments were done to prepare the materials by washing repeatedly with alcohol (Chevallier *et al*, *loc cit*)   The materials thus freed from pigments and fatty acids were dissolved in ether and examined spectrographically   The results obtained by this method for some fish oils agree closely with those for their unsaponifiable fractions   The method was, however, quite unsatisfactory for butter and ghee   That this method is not suitable for milk fats has been noted in a previous communication (De, *loc cit*)

The spectrograph used was Hilger's all-metal medium-sized quartz spectrograph, E316, with internal wave-length scale, in conjunction with the *rotating* sector   The source of light was a spark (4 mm gap) between steel-tungsten electrodes   The voltage was 15,000, initial exposure 10 seconds , the liquid containers were 1 cm quartz cells

Absorption curves have been prepared for all, some are shown here for illustrative purposes (Figs 1 to 7)
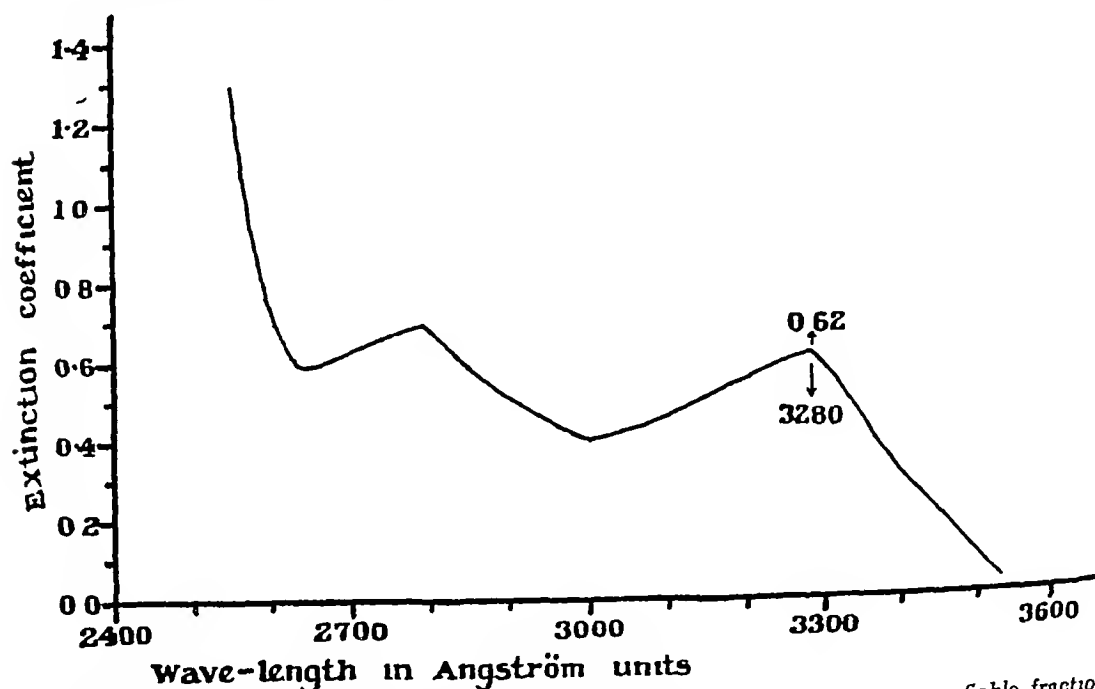
Fig 1   Absorption curve of 1 per cent solution of cod liver oil   The unsaponifiable fraction was extracted with ether
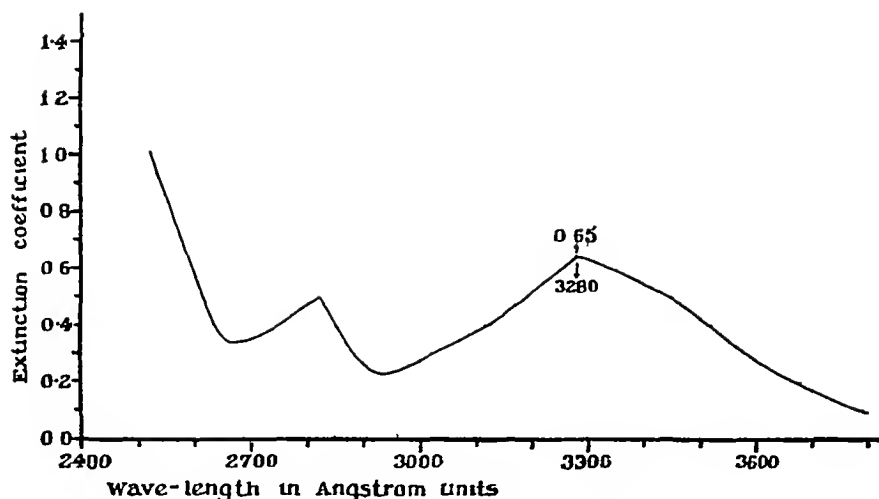
Fig 2   Absorption curve of 1 per cent solution of cod liver oil   The oil was re peatedly washed with alcohol and then dissolved in ether
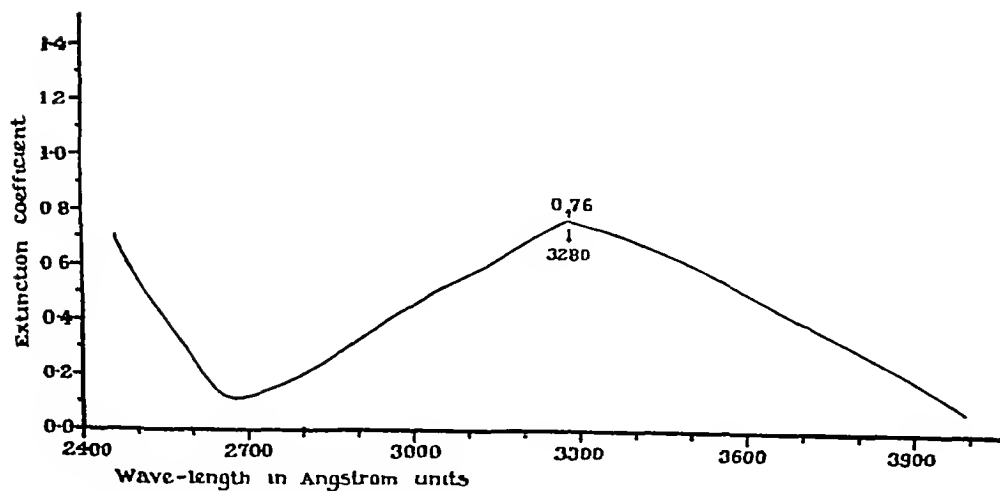
Fig 3   Absorption curve of halibut oil (1 capsule in 400 c c of ether)   The unsaponifiable fraction was extracted with ether
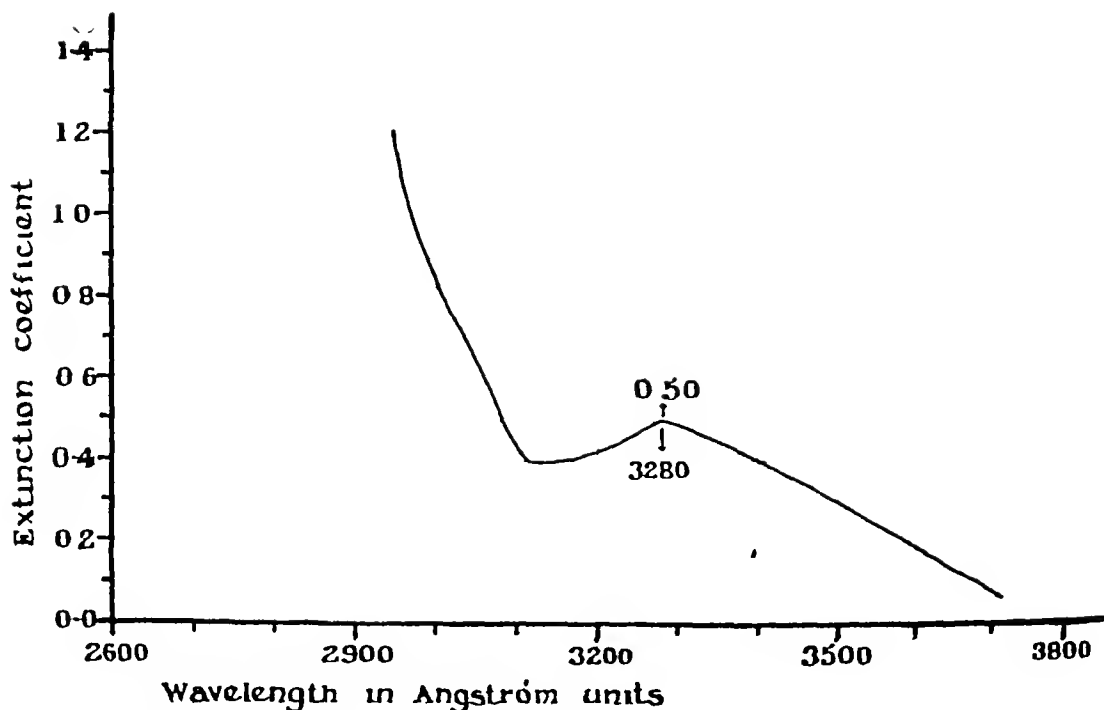
Fig 4   Absorption curve of 3 3 per cent solution of sardine fish oil   The unsaponifiable fraction was extracted with ether
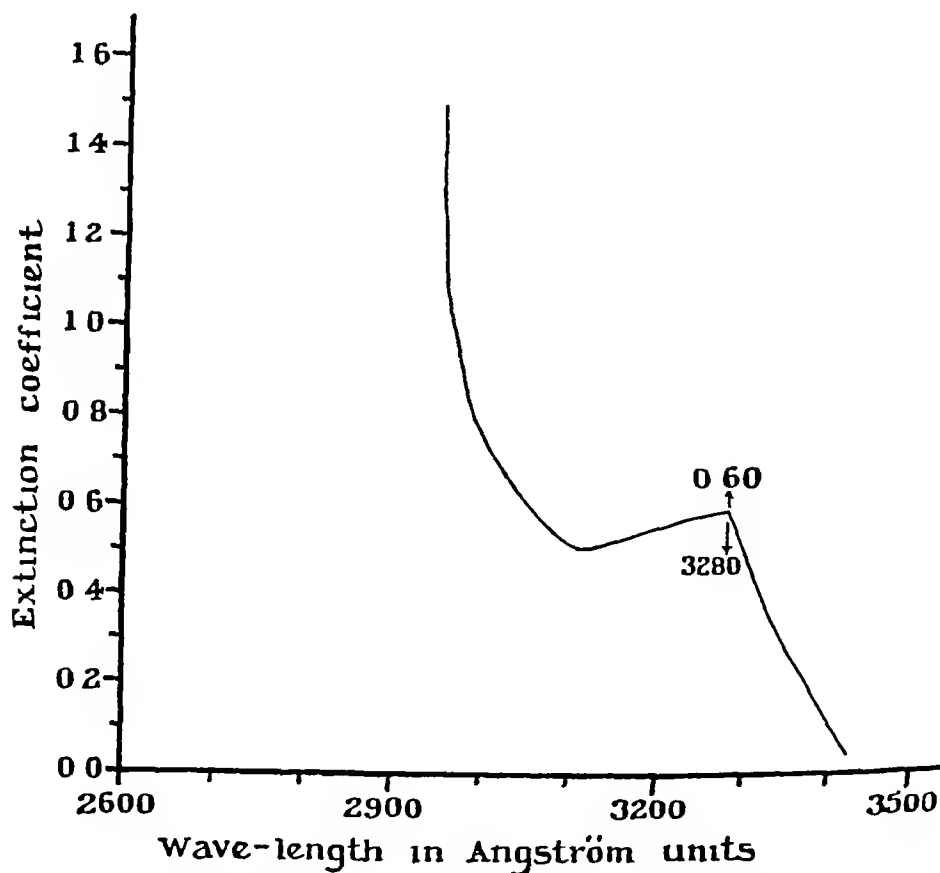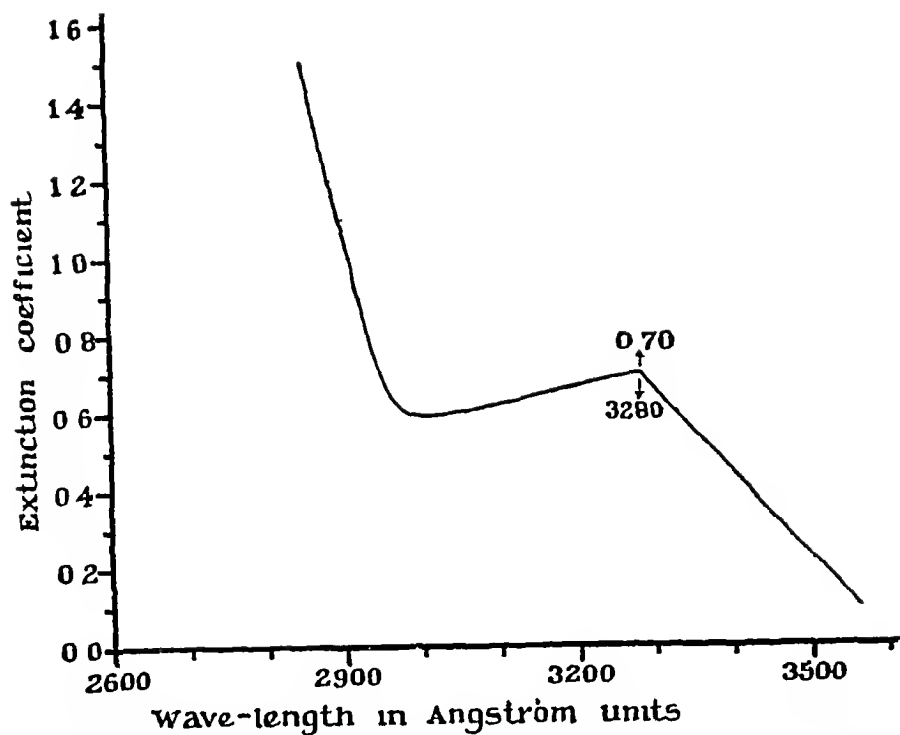

Fig 5   Absorption curve of 4 per cent solution of sardine fish oil   The oil was first repeatedly washed with alcohol and then dissolved in ether

Fig 6   Absorption curve of butter, 30 per cent solution in ether   The unsaponifiable fraction was extracted with ether
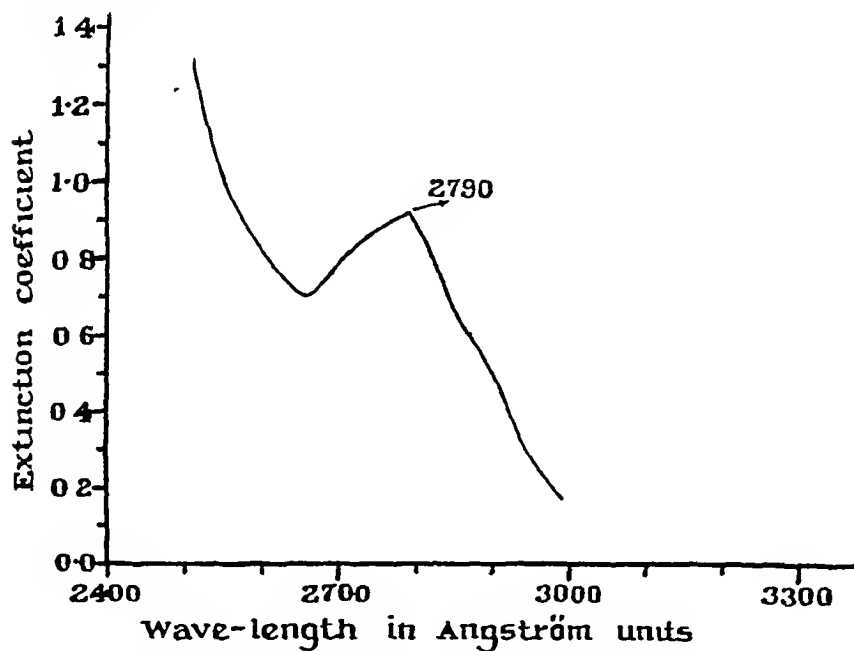
Fig 7   Absorption curve of 1 per cent solution of olive oil   The unsaponifiable fraction was extracted with ether

The Table gives all the observed data   column 6 gives the vitamin A content in absolute units and column 7 in rat units   It is to be noted that the figures in columns 5 and 7 agree —

TABLE.

Number of observations	Materials	Sources	Percentage of the solutions spectrographed	Observed value of the extinction coefficient at wave length 3,280 A U	Vitamin A content of the materials reported previously from biological tests, rat units per 100 g	Calculated vitamin A content of the materials in absolute units, γ per 100 g	Calculated vitamin content of the materials in rat units per 100 g
1	Cod-liver oil Unsaponifiable fraction	Norwegian (W E Smith & Co, Dispensing Department Spencer's, Madras)	1 0	0 64 (mean of 0 62 and 0 66)	54,545 0	40,000 0	133,333 3
2	Do, washed repeatedly with alcohol Unsaponifiable	do	1 0	0 65		40,625 0	135,416 7
3	Halibut oil Unsaponifiable fraction	Halverol capsules Parke, Davis & Co, Detroit, Mich, U S A	Content of 1 capsule in 400 0 0 ether	0 76	5,500 rat units per capsule or 3,200,000 rat units per 100 g minimum	1,000 0 per capsule	6,333 3] per capsule or 3,684,840 6 per 100 g
4	Sardine fish oil Unsaponifiable fraction	Government Fishery Station, Tanur	3 3	0 50	---	9,469 7	31,565 7
5	Do, after repeatedly washing with alcohol	do	4 0	0 60		9,375 0	31,250 0
6	Fish oil fraction Unsaponifiable	Fisheries Dept, Bombay	4 0	0 40		6,250 0	20,833 3
7	Lizard oil fraction Unsaponifiable	Collected from Northern India by Rai Sahib Dr Mula Singh of these Laboratories	. 1 0	0 36		22,500 0	75,000 0

No.	Substance	Source					
8	Shark oil Unsaponifiable fraction	Fisheries Dept , Tuti corin	0 3	0 80		166,666 6	555,555 5
9	Turtle oil Unsaponifiable fraction	do	6 0	0 24		2,500 0	8,333 3
10	Ghee Unsaponifiable fraction	Baby Health Week Association, Bombay	30 0	0 50		1,041 7	3,472 3
11	Butter Unsaponifiable fraction	Samples used in these Laboratories for feeding experiments	30 0	0 70	3,070 0	1,458 33	4,861 1
12	Milk Unsaponifiable fraction of fat				210 0	71 3	237 7* Average of cow, human, goat, and buffalo milk
13	Olive oil (Lucca oil)	Crosse and Blackwell (Mfg Co) Ltd , London	1 0	0 0	35 2	0 0	0 0
14	Gingelly oil	Coonoor market	5 0	0 0	0 0	0 0	0 0
15	Ceco nut oil	do	2 0	0 0	0 0	0 0	0 0
16	Mustard oil	do	2 0	0 0	0 0	0 0	0 0
17	Ground nut oil	do	2 0	0 0	0 0	0 0	0 0
18	Linseed oil	do	2 0	0 0	0 0	0 0	0 0
19	Cocogem	Tata Oil Mills Co, Ltd , Cochin State	2 0	0 0	0 0	0 0	0 0
20	Honey	W J F Barnes, Burnley, Victoria, Australia	5 0	0 0	0 0	0 0	0 0

* Quoted from the results reported in a previous communication (De, *loc cit*)

Discussion and Summary.

The results of the spectrophotometric analysis reported in this communication agree fairly well with available biological values None of the vegetable oils examined seemed to contain vitamin A in quantity sufficient to be detected by this method Considering the many drawbacks, difficulties and tediousness of biological tests, the spectrophotometric method, with the necessary precautions, seems to be a very valuable adjunct to biological methods of assay The findings may be summarized as follows —

(1) The vitamin A values for some oils and fats are given in absolute units and also in rat units

(2) The vegetable oils examined do not contain vitamin A in quantity sufficient to be detected by this method

(3) The method of using unsaponifiable fractions seems to be applicable universally

(4) The method of alcoholic washing was suitable for some fish oils only, while it was quite useless for milk fats

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OBSERVATIONS ON THE CHEMISTRY OF OXYTOCIN (THE OXYTOCIC HORMONE OF THE PITUITARY GLAND)

Part IV.

ACTION OF HNO₃, HNO₂ AND SO₂ EXTRACTIONS WITH PYRIDINE, ETHYL ALCOHOL, CHLOROFORM AND ACETONE

BY

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AND

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In continuation of our work we re-investigated the reaction of HNO_3, HNO_2 and SO_2 on the oxytocic hormone of the pituitary gland and the assay was carried out according to an improved technique   Solvents like pyridine, ethyl alcohol chloroform and acetone have been studied to find if the oxytocic hormone can be separated from some inactive materials by such means   Some of the results obtained are somewhat at variance with those obtained previously (Guha and Chakravorty, 1933)

EXPERIMENTAL

The material was prepared from bovine pituitary glands according to the method previously described (Guha and Chakravorty, *loc cit*)   The samples were assayed with some modifications in the method previously described by us (Das and Guha, 1934a and 1934b)   Strict care was observed in selecting the uteri from our laboratory-bred animals kept segregated from male guinea-pigs from a few days after their birth

(517)

Treatment with HNO_2 ($HCl+NaNO_2$) —To 5 c c of the pituitary extract solution four drops of concentrated HCl and seven drops of 30 per cent $NaNO_2$ (excess as tested by starch-iodide paper) were added with stirring   After a short interval it was heated on a water-bath for five minutes and ultimately evaporated to dryness under reduced pressure

The residue, which was yellow, was taken up in water and tested [P (128)]

A control was made with 5 c c of the same pituitary extract by adding four drops of concentrated HCl and heating on a water-bath for five minutes   The mixture was then evaporated to dryness under reduced pressure

The residue, which was pink, was taken up in water and tested [P (129)]

Treatment with HNO_2 ($CH_3COOH+NaNO_2$) —To 2 c c of the pituitary extract were added five drops of glacial acetic acid and three drops of 30 per cent $NaNO_2$, keeping the mixture cool with ice throughout the experiment   The mixture became turbid on addition of $NaNO_2$ It was allowed to remain in the ice-chest for about three minutes and ultimately dried in the vacuum desiccator over $CaCl_2$ and soda lime   The residue, which was yellow, was taken up in water and tested [P (134)]

A control was prepared by exactly the same method without the addition of sodium nitrite [P (135)]

Treatment with HNO_3 —Two c c of the pituitary extract were treated with nine drops of concentrated HNO_3 (Kahlbaum A R)   The solution became milky on addition of three drops of the acid but the precipitate dissolved on addition of more of the acid (Guha and Chakravorty, *loc cit*)   The mixture was allowed to evaporate to dryness in the vacuum desiccator over $CaCl_2$ and soda lime   The residue was taken up in water and tested [P (130)]

Treatment with SO_2 —Two c c of the extract were diluted to 5 c c and cooled in ice   The washed SO_2 gas from a generator ($Cu+H_2SO_4$) was passed into the cold solution for about six minutes   The solution which was saturated with SO_2 was allowed to remain in the ice-chest for thirty minutes and finally evaporated to dryness in a vacuum desiccator over $CaCl_2$ and soda lime   The residue was taken up in water and tested [P (158)]

Extraction with ethyl alcohol —Fifty mg of the dry powdered pituitary extract were heated under reflux with 3 c c of ethyl alcohol on a water-bath for fifteen minutes, filtered under suction while hot and the residue was washed with 1 c c of cold ethyl alcohol   The filtrate and washings were evaporated by means of a fan and finally in a vacuum desiccator over $CaCl_2$ and soda lime   The dry extract was taken up in water [P (142)]

The residue remaining after extraction with alcohol was dried in a vacuum desiccator and dissolved in water [P (143)]

Extraction with acetone —By exactly the same method extractions were carried out with acetone and the soluble and insoluble fractions were called P (146) and P (147) respectively

Extraction with chloroform —Fifty mg of the dry powdered pituitary extract were extracted with chloroform in the same way as above

The chloroform soluble and insoluble fractions were called P (144) and P (145) respectively

Prolonged extraction with chloroform —Fifty mg of dry powdered extract were refluxed with 3 c c of chloroform for one hour on a water-bath The mixture was filtered under suction and the residue washed with 1 c c of cold chloroform The filtrate was evaporated to dryness by means of a fan and the residue was taken up in warm water [P (150)]

The residue after extraction was freed from traces of chloroform and dissolved in warm water [P (151)]

Alkaline extraction with chloroform —Twenty-one mg of dry powdered extract were triturated in a mortar with 21 mg of sodium carbonate with a tiny drop of water to make a paste The paste was then extracted with 6 c c of cold chloroform and filtered The filtrate was evaporated to dryness by means of a fan and the small residue obtained was taken up in warm water [P (153)]

Extraction with pyridine —Fifty mg of the dry powdered pituitary extract were heated under reflux with 3 c c of pyridine for fifteen minutes on a water-bath, filtered under suction and the residue washed with 1 c c of cold pyridine The filtrate was evaporated to dryness in a vacuum desiccator over concentrated H_2SO_4 The dry residue free from the smell of pyridine was dissolved in warm water [P (148)]

The residue after extraction was also kept in the vacuum desiccator in order to remove traces of pyridine and was taken up in warm water [P (149)]

The results are given in Tables I and II —

TABLE I

Number of samples	Descriptions	Percentage of inactivation
P (128)	Treated with $HCl + NaNO_2$	100
P (129)	Control with HCl	About 75
P (134)	Treated with $CH_3COOH + NaNO_2$	85 to 90
P (135)	Control with CH_3COOH	0
P (158)	Treated with SO_2	50 to 60
P (130)	Treated with HNO_3	100

TABLE II

Number of samples	Descriptions	Percentage of activity retained
P (142)	Ethyl alcohol extract	0
P (143)	Residue after extraction with ethyl alcohol	100
P (144)	Chloroform extract (fifteen minutes' reflux)	0
P (145)	Residue after extraction with chloroform	100
P (150)	Chloroform extract (one hour's reflux)	0
P (151)	Residue after extraction with chloroform	100
P (153)	Chloroform extract in presence of Na_2CO_3	0
P (146)	Acetone extract	0
P (147)	Residue after extraction with acetone	100
P (148)	Pyridine extract	0
P (149)	Residue after extraction with pyridine	100

SUMMARY

(1) Pyridine, ethyl alcohol, chloroform and acetone have not been able to dissolve or inactivate the oxytocic hormone under our experimental conditions

(2) Nitrous acid in the presence of concentrated HCl completely inactivates the oxytocic hormone while concentrated HCl itself has been found to inactivate the hormone considerably

(3) Nitrous acid in presence of glacial acetic acid has been found to inactivate the hormone to the extent of 85 to 90 per cent under our experimental conditions, while glacial acetic acid by itself has practically no effect on the activity

(4) Nitric acid inactivates the hormone completely

(5) SO_2 inactivates the hormone by 50 to 60 per cent

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VITAMIN A VALUES OF INDIAN FISH LIVER OILS DETERMINED BIOLOGICALLY AND TINTOMETRICALLY

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In previous papers (Chakravorty, Mookerji and Guha, 1933, Ghosh, Chakravorty and Guha, 1933) it has been shown that the liver oils of some common fishes of Bengal are rich in vitamin A, as determined by the tintometric method, most of them being richer than cod-liver oil but less potent than ordinary specimens of halibut-liver oil   It was pointed out, however, that the values determined biologically in preliminary experiments did not agree well with the Carr-Price blue values of the oils and it was suspected that some other chromogen besides vitamin A might be present in those oils (Chakravorty, Mookerji and Guha, *loc cit*)   Some of the oils have since been investigated in detail biologically and tintometrically and the results are recorded in the present paper

The problem of the relative reliability of the biological, tintometric and spectrographic methods for the estimation of vitamin A has been the subject of several investigations during recent years and attempts have also been made to increase the accuracy of these methods (Coward, Dyer, Morton and Gaddum, 1931, Coward, Dyer and Morton, 1932)   But though much advance has been made, the problem still presents difficulties   The results obtained with the Indian fish liver oils display some but not good agreement between the blue values and biological values   A strict comparison is, however, rendered difficult because of the large individual variations observed in the biological method itself, which has been especially investigated by Coward *et al* (*loc cit*)

Dann (1932) has investigated the stability of vitamin A in different solvents, and Dyer, Key and Coward (1934) have lately shown that the biological activity

of the international standard of carotene depends to a great extent on the solvent used. Our work also shows that the observed biological potency of the Indian fish liver oils varies greatly with the diluent used. Alcohol, ethyl acetate, chloroform and arachis oil have been used as vehicles for the administration of the oils.

Experimental

The biological assay of vitamin A in the fish liver oils was carried out according to the technique described before (Ghosh and Guha, 1934). The liver oils were extracted in the usual way by grinding up the livers with anhydrous sodium sulphate followed by extraction with cold chloroform.

The diluents used for the liver oils were ethyl alcohol, ethyl acetate, chloroform and arachis oil, of which the first did not dissolve the oils completely. The first three solvents were blown off by means of a fan at room temparature just before administration to the animals. Arachis oil preparations were fed as such.

The standard used for comparison was a sample of the international standard of carotene, which we dissolved in ethyl acetate. The potency of the carotene was, however, found to be considerably less than it was supposed to be. The figures for the different oils were computed by comparison with this sample of carotene and are given in Table I. These values are comparable among themselves, though the absolute values in terms of international units would probably be different if a fresh and more potent sample of the international standard of carotene were available.

All the rats used were females except rats Nos. 597, 442, 498, 605, 359, 406, 411 and 497. No attempt has been made to take the sex factor into account.

TABLE I

Names of preparations and diluents	Rat number	Daily dose (in mg.)	Expt period (in weeks)	Weekly average growth (in g.)	Mean weekly average growth (in g.)	International units of vitamin A (per g.)
*K L O (7) (absolute alcohol)	597	40	3	8	7	583
	606	40	3	0		
	609	40	4	12		
	614	40	3	4		
	635	40	3	4		
	636	40	3	4		
	640	40	3	6		

*K L O = *Katla*-liver oil (*Catla catla*). All K L O preparations were made from K L O (5)

TABLE I —*contd*

Names of prepa rations and diluents	Rat number	Daily dose (in mg)	Expt period (in weeks)	Weekly average growth (in g)	Mean weekly average growth (in g)	International units of vitamin A (per g)
K L O (6) (ethyl acetate)	554	40	3 5	4 5		
	558	40	3 5	0		
	561	40	4	3	5 5	458
	588	40	3 5	10		
	595	40	3 5	4 5		
K L O (8) (chloroform)	639	36	3	0		
	641	36	3	0		
	642	36	3	6	4 3	397
	645	36	3	4		
	651	36	3	4		
	681	36	3	3 3		
K L O (9) (arachis oil)	636	36	3	1		
	639	36	3	2		
	640	36	3	0	2 5	231
	641	36	3	0		
	642	36	3	3		
	680	36	3	4		
* R L O (9) (ab solute alcohol)	437	40	3	7		
	442	40	3	3		
	505	40	3 5	6		
	492	40	2 5	8	5 7	475
	493	40	2 5	5		
	502	40	2 5	0		
	507	40	2 5	5		

* R L O = *Rohit* liver oil (*Labeo rohita*) All R L O preparations were made from R L O (8)

Vitamin A Values of Indian Fish Liver Oils.

TABLE I—contd

Names of preparations and diluents	Rat number	Daily dose (in mg)	Expt period (in weeks)	Weekly average growth (in g)	Mean weekly average growth (in g)	International units of vitamin A (per g)
R L O (12) (ethyl acetate)	551	40	4	5		
	561	40	4	2 5		
	593	10	4	5		
	596	40	4	4	4 4	368
	601	40	4	5		
	602	40	4	5		
R L O (10) (chloroform)	406	20	3 5	1		
	498	20	3 5	4		
	500	20	3 5	1	1 6	265
	504	20	3 5	1		
	495	20	3 5	1		
R L O (14) (arachis oil)	645	36	3	2		
	653	36	3	0		
	677	36	3	3	2	186
	678	36	3	1		
	679	36	3	2		
*M L O (5) (absolute alcohol)	594	36	4	0		
	606	36	3 5	8		
	607	36	4	0		
	611	36	3	3	4 8	444
	612	36	3	3		
	634	36	3	5		
	654	36	3	5		

*M L O = Mrigal-liver oil (Cirrhina mrigala) All M L O preparations were made from
M L O (3)

TABLE I—*concld*

Names of preparations and diluents	Rat number	Daily dose (in mg)	Expt period (in weeks)	Weekly average growth (in g)	Mean weekly average growth (in g)	International units of vitamin A (per g)
M L O (4) (ethyl acetate)	602	36	4	0	4 1	381
	605	36	4	0		
	616	36	4	2 5		
	639	36	4	3		
	642	36	4	4		
	652	36	4	7		
M L O (6) (chloroform)	592	36	3 5	0	2 5	231
	640	36	3 5	1 7		
	646	36	3 5	3 1		
	649	36	3 5	0		
	653	36	3 5	2		
	676	36	3 5	4		
	677	36	3 5	2 6		
	678	36	3 5	1 4		
M L O (2) (arachis oil)	359	30	3	3	1 8	200
	406	30	3	1		
	410	30	3	2		
	411	30	3	1		
	436	30	3	0		
* V L O (3) (absolute alcohol)	494	22	3 5	4	6	909
	495	22	3 5	5		
	498	22	3 5	6		
	496	22	3 5	4		
	497	22	3 5	10		
	500	22	3 5	4		
	506	22	3 5	8		
V L O (4) (ethyl acetate)	493	36	3	7	5	464
	494	36	3	3		
	505	36	3	3		
	524	36	3	7		
V L O (5) (chloroform)	506	36	3 5	4	3 5	325
	507	36	3 5	3		

* V L O = *Veth liver oil (Lates calcarifer)* All V L O preparations were made from V L O (2)

The tintometric estimation of the oils was carried out in the usual manner with the Lovibond tintometer of the British Drug Houses pattern (Chakravorty, Mookerji and Guha, *loc cit*) The dilutions of the oils in chloroform were so arranged as to give in general blue unit figures between 4 and 8, the region where the relation between dilution and the intensity of the blue colour produced is generally held to be most nearly linear The Carr-Price values of the oils are given in Table II —

TABLE II.

Extract number	Mg oil in 2 2 c c reaction mixture	Colour produced by the oils with SbCl₃			Carr-Price value
		B	Y	N	
M L O (3)	1 1	4	2 1	0 4	
	1 2	4 9	2 1	0 4	
	1 3	5 8	2 8	0 4	175
	1 5	7 2	3 3	0 4	
	1 4	6 5	2 8	0 4	
K L O (5)	0 7	3 4	2	0 5	
	1 1	4 6	1 8	0 5	
	1 4	5 4	2 3	0 5	157
	1 7	6 1	2 4	0 5	
	2	7 3	2 4	0 5	
R L O (8)	0 8	3 9	1 2	0 4	
	1	5	2 2	0 4	189
	1 5	6 6	2 2	0 4	
V L O (2)	1 0	7 5	2 3	0 2	
	0 9	6 6	2 3	0 4	293
	0 8	5 3	2 1	0 4	

Table III gives a comparison of the biological values obtained with the oils (ethyl alcohol being used as the diluent) and their corresponding Carr-Price values The ratios of the activities of *Mrigal-, Rohit-, Katla-* and *Vethi-*liver oils are of the

order 1 1 1 1 3 2 0 as determined biologically and of the order 1 1 1·0 9 1 7 as estimated tintometrically —

<div align="center">TABLE III.</div>

Extract number	Biological tests (international units)	Colorimetric tests (Carr-Price value)
M L O (3)	444	175
R L O (8)	475	189
K L O (5)	583	157
V L O (2)	909	293

DISCUSSION

Table III shows that there is some agreement between the values obtained by the biological method of assay and those obtained by the tintometric method The agreement, however, is not strict The order of accuracy of the biological method of assay is itself, however, not high, as has generally been observed, because of fairly large individual variations A strict comparison between the biological and tintometric methods is, therefore, difficult Some further investigations in progress, especially with the non-saponifiable fractions of the oils, are expected to throw some light on the question as to the presence of some other chromogen or chromogens besides vitamin A in these Indian fish liver oils Datta and Banerjee (1934) have lately investigated some Indian fish oils tintometrically and biologically and they report very good agreement between the biological and tintometric values

SUMMARY

The same samples of the liver oils of *Katla, Rohit, Mrigal* and *Velki* fishes have been investigated for vitamin A content both biologically and tintometrically There is some but not strict agreement between the biological and tintometric values obtained

The biological activities of each of the oils in relation to the diluents used are of the following order ethyl alcohol, ethyl acetate, chloroform and arachis oil (the potency of *Velki*-liver oil in arachis oil was not investigated)

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DETOXICATION OF SNAKE VENOM BY THE PHOTODYNAMIC ACTION OF METHYLENE BLUE

BY

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Recent work on the photodynamic action of methylene blue in inactivating bacteriophage and the infective agents of various virus diseases (Clifton, 1931, Perdrau and Todd, 1933, 1933a, Shortt and Brooks, 1933) led us to consider the possibility that a similar action might be brought about by exposing the snake venoms to the action of methylene blue in the presence of light

Should such be the case it envisaged the further possibility that such inactivated venoms might profitably be used as antigenic agents for the rapid production of anti-bodies in a manner similar to the use of formalized venoms for the same purpose

The fact that methylene blue, if it is going to act, does so quickly, promised a great saving in time in the production of a detoxicated venom

MATERIALS AND METHODS

For the purposes of our investigation it was decided to use *Daboia* venom because the methods used by us in titrating this product in the routine of antivenene production enable the results of any experiment to be read in a few minutes, thereby saving much time in repeating observations

The experimental animals used were standard pigeons weighing between 290 and 310 grammes The procedure adopted was first to ascertain the minimum lethal dose of the sample of dried *Daboia* venom for the standard pigeons From this information a solution of the venom in distilled water was prepared in the required strength

The methylene blue solution was similarly prepared in the strength decided upon for the particular experiment

The venom solution and the methylene blue were mixed and poured into a large Petri dish placed on white paper and exposed to sunlight   The size of the Petri dish was so adjusted that the depth of the mixture did not exceed about three millimetres   A sample of the mixture was kept in the dark as a control   At the intervals required by the particular experiment the mixture of venom and methylene blue was injected intravenously into pigeons and the death or survival of the latter noted   Results could be read in fifteen minutes as a pigeon which was going to die never survived this period

The following experiments were carried out —

(a) *Experiment to determine whether* Daboia *venom was detoxicated by the photodynamic action of methylene blue*

EXPERIMENT

The minimum lethal dose of *Daboia* venom was first determined to be 0 03 mg A 1 in 50,000 solution of methylene blue was prepared and mixed with equal parts of *Daboia* venom solutions of different strengths so adjusted as to obtain final mixtures containing 2, 3, 4, 5, 8, 12 and 20 minimum lethal doses of *Daboia* venom in 1 c c of 1 in 100,000 solution of methylene blue   The mixtures were exposed to sunlight for 20 minutes and 1 c c from each injected intravenously into standard pigeons   Control mixtures of similar strength were kept in the dark   The results are tabulated below —

TABLE I

Showing the detoxicating effect of the photodynamic action of methylene blue on Daboia *venom*

Number of minimum lethal doses of *Daboia* venom in 1 c c	Concentration of methylene blue in mixture	Time of exposure to sunlight in minutes	Method of injection of mixture	Effect on pigeons	REMARKS
2	1 in 100,000	20	Intravenous	Survived	One c c of the control mixture containing 2 minimum lethal doses kept in the dark for 20 minutes killed a standard pigeon Two minimum lethal doses of *Daboia* venom exposed to sunlight for 20 minutes also killed a standard pigeon
3	,,	,,	,,	,,	
4	,,	,,	,,	,,	
5	,,	,	,,	,,	
8	,,	,,	,,	,,	
12	,	,,	,,	,,	
20	,,		,	,	

This experiment proved that the photodynamic action of methylene blue has a detoxicating action on *Daboia* venom

(b) *Experiment to determine the effect of variation in the time of exposure on the photodynamic action of methylene blue on* Daboia *venom in a given mixture*

EXPERIMENT -

A mixture containing 80 minimum lethal doses of *Daboia* venom per c c in 1 in 100,000 solution of methylene blue was prepared and exposed to sunlight One c c or the mixture was injected intravenously into standard pigeons at short intervals The results are detailed in Table II —

TABLE II

Showing the effect of variations in the time of exposure on the photodynamic action of methylene blue on Daboia *venom*

Number of minimum lethal doses of *Daboia* venom in 1 c c	Concentration of methylene blue in mixture	Time of exposure to sunlight in minutes and seconds	Method of injection of mixture	Effect on pigeons	REMARKS
80	1 in 100,000	1–45	Intravenous	Died	One c c of the mixture kept in the dark as a control for 24 hours killed a standard pigeon
,,	,	6–10	,	,,	
,	,,	10–45	,,	Survived	
,	,,	17–0	,,	,,	

This experiment proved that complete detoxication is not immediate

(c) *Experiment to determine the effect on the photodynamic action of methylene blue of variations in the strength of the* Daboia *venom solution, the strength of the methylene blue solution remaining constant*

EXPERIMENT

Two mixtures were prepared containing respectively 10 and 100 minimum lethal doses of *Daboia* venom per c c in 1 in 100,000 solution of methylene blue These mixtures were exposed to diffused light and standard pigeons

were inoculated intravenously at short intervals The results are detailed in Table III —

<div align="center">TABLE III</div>

Showing the effect on the photodynamic action of methylene blue of variation in the strength of the Daboia *venom solution*
(two pigeons for each time interval)

Number of minimum lethal doses of *Daboia* venom in 1 c c of mixture	Concentration of methylene blue in mixture	Time of exposure to sunlight in minutes and seconds	Method of injection of mixture	EFFECT ON PIGEONS	
				(1)	(2)
10	1 in 100,000	1–0	Intravenous	Died	Survived
,,	,,	2–0	,,	,,	Died
,,	,,	3–0	,,	Survived	Survived
,,	,,	3–9	,,	,,	,,
100	,,	3–0	,,	Died	Died
,,	,,	5–0	,,	,,	,,
,,	,,	11–0	,,	,,	,,
,,	,,	14–0	,,	,,	,
,,	,,	18–0	,,	,,	,,
,,	,,	21–0	,	,,	Survived
,,	,,	23–0	,,	,,	,,
,,	,	26–0	,,	,,	,,
,,		29–0	,	Survived	,,

This experiment showed that increasing the amount of the *Daboia* venom in the solution lengthened the time for complete detoxication of the solution

(d) *Experiments to determine the effect on the photodynamic action of methylene blue of variations in the strength of the methylene blue solution, the strength of the* Daboia *venom solution remaining constant*

<div align="center">EXPERIMENT (A)</div>

Three mixtures were prepared each containing 10 minimum lethal doses of *Daboia* venom per c c but the strength of the methylene blue solution in each varied, being 1 in 10,000, 1 in 100,000 and 1 in 1,000,000, respectively These mixtures

were exposed to sunlight and standard pigeons were inoculated at intervals   The results are detailed in Table IV   For the sake of simplicity only essential details are included in the table, the methods being the same as for previous experiments —

TABLE IV

Showing the effect on the photodynamic action of methylene blue on Daboia *venom of variations in the strength of the methylene blue solution*

METHYLENE BLUE A 1 IN 10,000		METHYLENE BLUE B 1 IN 100,000		METHYLENE BLUE C 1 IN 1,000,000	
Exposure to sunlight in minutes and seconds	Result	Exposure to sunlight in minutes and seconds	Result	Exposure to sunlight in minutes and seconds	Result
1–25	Died	1–30	Died	1–20	Died
3–35	,,	3–30	,,	3–20	,
6–15	,,	8–30	,	6–25	,,
12–30	,,	12–30	,,	12–20	,,
16–5	,,	15–30	,,	15–20	,,
20–30	,,	20–30	,	20–20	,,
30–0	,,	26–40		37–0	,,
34 35	Survived	35–45	,	49–25	,,
39–0	,	39–0	,,	60–0	,,
		42–10	,,	65–0	,,
		46–35			
		53–20	Survived		
		55–0	,		

This experiment showed that of the three strengths of methylene blue solution the photodynamic action was strongest in the 1 in 10,000 solution

EXPERIMENT (B)

Three mixtures were prepared each containing 20 minimum lethal doses of *Daboia* venom per c c but the strength of the methylene blue solution in each varied

being 1 in 5,000, 1 in 50,000 and 1 in 75,000, respectively   The rest of the experiment was on the lines detailed for Experiment (A)   The results are detailed in Table V —

TABLE V

Showing the effect on the photodynamic action of methylene blue on
Daboia *venom of variations in the strength of the*
methylene blue solution

METHYLENE BLUE A 1 IN 5,000		METHYLENE BLUE B 1 IN 50,000		METHYLENE BLUE C 1 IN 75,000	
Exposure to sunlight in minutes and seconds	Result	Exposure to sunlight in minutes and seconds	Result	Exposure to sunlight in minutes and seconds	Result
2–4	Died	2–30	Died	2–25	Died
4–10	,,	4–25	,,	4–15	,,
8–28	,,	8–20	,,	8–30	,,
11–20	,,	11–50	,,	11–20	,,
16–45	,,	14–20	,,	16–35	,,
19–30	,,	16–40	Survived	19–30	Survived
24–45	,,	20–20	,	23–5	,
28–20	Survived	23–5	,,		
30–0	,,				

This experiment showed that of the three strengths of methylene blue solution the photodynamic action was strongest in the 1 in 50,000 solution

EXPERIMENT (C)

Three mixtures were prepared each containing 20 minimum lethal doses of *Daboia* venom per c c but the strength of the methylene blue solution in each varied

The immunization was carried on for over ten weeks, each goat receiving approximately 500 mg of detoxicated venom   At the end of this period the sera of all the goats were tested against *Daboia* venom but none showed any anti-toxic value   It was, however, interesting to find some neutralizing value in the serum of one goat which had on one occasion received incompletely detoxicated venom and was definitely ill with some local reaction at the site of injection

It would appear from the above that the detoxication of *Daboia* venom by the photodynamic action of methylene blue completely destroys its antigenic properties

Summary and conclusions

1   *Daboia* venom can be rapidly and completely detoxicated by the photodynamic action of methylene blue

2   The rapidity of the photodynamic action is affected by variations in the time of exposure to light, the amount of *Daboia* venom present in mixtures and the concentration of methylene blue in the solutions used

3   The optimum concentration of methylene blue under the conditions of the present experiments was in the neighbourhood of 1 in 50,000

4   The detoxication of *Daboia* venom by the photodynamic action of methylene blue destroys its antigenic properties

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ANTI-RABIC IMMUNIZATION   PROBABLE LINES OF PROGRESS IN IMPROVEMENT OF METHODS

BY

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SINCE the introduction of Pasteur's dried cord method of immunization against rabies a multiplicity of techniques has been introduced with the common object in view of improving upon the original method and lowering the mortality among the population at risk   In spite, however, of the most efficacious of these methods there is still a considerable mortality among the severely bitten section of those coming for anti-rabic treatment and any method which will reduce this mortality, even in a minor degree, should be fully exploited

The methods of anti-rabic immunization in vogue at present may be summarized briefly under three heads —

   (a) Methods involving the use of dead virus
   (b) Methods involving the use of attenuated live virus
   (c) Methods involving the use of virus, dead or attenuated, combined with anti-rabic serum

In a search for methods which would give results superior to any of the techniques comprised within the methods mentioned above we have been led to study anew the value of rabicidal serum and of vaccines containing live fixed virus in no way attenuated   It is the results of these researches which form the subject of this communication and we offer them in the hope that they will lead others to extend our work by investigation along similar lines

For convenience of description we may deal with our researches on this subject under four heads —

   1   The method of preparation and standardization of anti-rabic serum

2 The use of anti-rabic serum as an adjunct to treatment by carbolized dead virus

3 Treatment by one dose of a combination of fresh living fixed virus and anti-rabic serum

4 The use of fresh living fixed virus as an adjunct to treatment by carbolized dead virus

1 THE METHOD OF PREPARATION AND STANDARDIZATION OF ANTI-RABIC SERUM

The question of the possibility of producing an efficacious anti-rabic serum is an old one and necessarily much of our preliminary work has been merely a repetition of experiments previously performed by other workers, the majority of whom, for unstated reasons, have abandoned the use of serum while claiming encouraging results from its use

Babés and Lepp (1889), Marie (1905), Remlinger (1905), Schnurer (1905) and Semple (1908) have all proved the presence of a rabicidal element in anti-rabic serum *in vitro* but opinions as to its prophylactic value *in vivo* differ widely

As long ago as 1889 Babés and Lepp claimed to have successfully protected animals against subsequent rabies infection by injecting them with the blood of immunized dogs   Semple (1903) using an anti-rabic serum from a highly immunized horse had good results and more recently Ponomareff and Solovieff (1928) have described a new method of preparing a vaccine said to be particularly useful for the production of anti-rabic serum   Fermi (1909) states that horse immune serum possesses the most powerful rabicidal action and next in order of potency mentions the serum obtained from asses, sheep and dogs

The animals used by us for the preparation of anti-rabic serum have been buffaloes and sheep   The results with buffalo serum were disappointing and the experiments with which we are concerned in this account all refer to sheep serum

Immunization of sheep for the production of anti-rabic serum

Immunization of the sheep was carried out according to the plan detailed below, the virus in all cases being the Paris fixed virus —

(a) Ten c c of sheep carbolized 5 per cent dead vaccine daily for three weeks

(b) One week's rest

(c) Ten c c of (a) daily for three weeks

(d) One week's rest

(e) Ten c c of (a) daily for three weeks

(f) One week's rest

(g) Ten c c of sheep carbolized 5 per cent attenuated live virus daily for one week (virus is incubated at 37°C with 1 per cent carbolic for 12 hours, i e , half the time in routine use for the ordinary dead vaccine)

(h) One week's rest

(i) Ten c c of 1 per cent fresh live fixed virus sheep's brain in distilled water, three doses with two days' interval between doses

(j) One week's rest

The total period occupied in this course of immunization is 105 days and at this stage the titre of the serum has usually reached the standard adopted by us as a minimum   This standard and the method of titration will be dealt with later   If the titre is found to be satisfactory the immunized sheep are bled at weekly intervals (240 c c at a bleeding) for a month and then rested for two months   Subsequent courses of hyper-immunization are given when necessary as under —

(a) Ten c c of (a) as in original immunization

(b) One week's rest

(c) Ten c c of (q) as in original immunization

(d) One week's rest

(e) Ten c c of (i) as in original immunization

(f) One week's rest

Titration of serum

The only method we have used so far in titration is by complement-fixation tests   We do not consider that the results of complement-fixation tests necessarily indicate with any degree of accuracy the potency of the serum therapeutically—for this biological tests are necessary—but complement-fixation tests have proved of value as a guide to the selection of suitable sera

The complement-fixation tests were carried out in accordance with method No 4 of the Medical Research Council, England   The technique is so well known that it needs no further mention, but some comment on the antigen used is necessary   For complement fresh guinea-pig serum obtained on the day of the test was used   The antigen was the filtrate from carbolized 5 per cent Paris virus sheep vaccine over a month old   The reason for not using fresh vaccine was that from a series of complement-fixation tests carried out to determine the antigenic value of the filtrate from carbolized vaccine the observation has been made that there is a definite improvement in the antigenic value a month after the manufacture of the vaccine, as compared with fresh vaccine, and that this higher value is maintained constant for at least five months

The standard to which the anti-rabic sera must conform was decided by us arbitrarily   It was fixed at a level where the serum in a 1 in 5 dilution was capable of completely fixing not less than 8 minimum haemolytic doses of complement   The titre usually obtained was between 8 and 12 M H D

2   THE USE OF ANTI-RABIC SERUM AS AN ADJUNCT TO TREATMENT BY CARBOLIZED DEAD VIRUS

(a) Animal experiments

These experiments may be conveniently divided into two series —

(1) Experiments to test the neutralizing action *in vitro*, if any, of anti-rabic serum on fresh rabies fixed virus

(2) Experiments to test the value of immune serum as an adjunct to treatment with carbolized dead virus

(1) *Experiments to test the neutralizing action* in vitro, *if any, of
anti-rabic serum on fresh rabies fixed virus*

Experiment No 65 —In this experiment a 5 per cent emulsion of Paris fixed
virus was prepared   To this emulsion an equal quantity of anti-rabic serum was
added and the mixture incubated at 37°C   0 2 c c of this mixture was injected sub-
durally into groups of rabbits (two in each group) immediately and at intervals of
1, 2, 4 and 8 hours   Two control animals were given 0 2 c c of a 2 5 per cent
emulsion of the same fixed virus in normal saline incubated at 37°C for 4 hours
and four further controls (two in each group) were given equal parts of normal sheep
serum plus 5 per cent emulsion of the same fixed virus which had been incubated
at 37°C for 4 and 8 hours respectively

Experiment No 65(*a*) —This experiment was conducted on similar lines   The
combined results of the two experiments are given in Table I —

TABLE I

Showing the results of experiments to test the neutralizing action in vitro,
if any, of anti-rabic serum on fresh rabies fixed virus

Group	Number of rabbits	Period of contact of virus and serum	Died of rabies	Escaped	Died of other causes	Average period to death in days
A	6	Immediate	5	1		11 20
B	6	1 hour	4	1	1	10 75
C	6	2 hours	5	1		11 60
D	6	4 ,,	5	1		11 20
E	6	8 ,,	5	1		12 40
CONTROLS						
F	6	8 hours with normal saline	6			9 50
G	6	4 hours with normal serum	6			10 00
H	6	8 hours with normal serum	6	..	1	9 80

Five out of the thirty rabbits given virus plus immune serum escaped infection
and the twenty-four animals which died of rabies had an average 'period to
death' of 11 42 days   One rabbit died of 'other causes'

Seventeen out of the eighteen controls died of rabies with an average 'period
to death' of 9 61 days

These experiments show that the anti-rabic serum does not exhibit complete rabicidal action in 8 hours but there is no doubt that the fixed virus is attenuated as proved by the prolongation of the 'period to death'

It has to be borne in mind that the test—subdural infection—is a very severe one

Experiment No 65(*b*) —This experiment is a repetition of experiments Nos 65 and 65(*a*) with modifications introduced by the use not only of whole anti-rabic serum but of various serum fractions and their mixtures   The fractionation of the serum was carried out by the standard ammonium sulphate method as used at the Lister Institute*   In the experiments the rabicidal action *in vitro* of immune whole serum, the pseudo-globulin fraction, the eu-globulin fraction, and a mixture of the two latter was tested   Thirty-two rabbits were divided into four groups to be tested with the whole serum, the eu-globulin, the pseudo-globulin and the combine globulins, respectively   All the globulin fractions before mixing with the lived virus emulsion were reduced to a common degree of concentration with 1 per cent saline solution   This was done in order to get strictly comparable results with the various fractions

In other respects the experiment was carried out on the lines of those previously dealt with

TABLE II

Showing the results of experiments to test the neutralizing action in vitro, if any, of whole anti-rabic serum and fractions thereof on fresh rabies fixed virus

Hours in incubator	Type of serum	Group	Result	Period to death in days	REMARKS
1		A	Rabies	14	
1		A	Survived		
2		B	Rabies	13	
2	Unconcentrated serum	B	Survived		
4		C	Rabies	11	Average ' period to death ' 12 4 days
4		C	,,	12	
8		D	,,	12	
8		D	Survived		

* We are indebted to the Director Dr J C G Ledingham, F R S , for sending us complete particulars of this method and to Lieut Colonel J Taylor, D S O , I M S , for permitting us to study the actual method in practice as used at the Central Research Institute, Kasauli, in the concentration of antivenene

Table II—*concld*

Hours in incubator	Type of serum	Group	Result	Period to death in days	Remarks
1		A1	Survived		
1		A1	,,		
2		B1	,		
2		B1			
4	Eu globulins	C1	,		All Survived
4		C1			
8		D1	,,		
8		D1	,		
1		A2	Survived		
1		A2	,,		
2		B2	,		
2		B2	Rabies	14	
4	Pseudo globulins	C2	Survived		Passage, positive
4		C2	,,		
8		D2	,,		
8		D2	,		
1		A3	Rabies	21	
1		A3	Survived		
2		B3	Rabies	11	
2		B3	Survived		
4	Combined globulins	C3	,		Average ' period to death' 16 days
4		C3	,,		
8		D3	,		
8		D3			

dead virus certain interesting points have emerged   These points will now be taken up —

1   There is a striking demonstration that the use of anti-rabic serum at the end of the process of immunization with carbolized dead virus has a most disadvantageous effect and definitely increases the risk of contracting rabies, almost as though it sensitized the subject to the street virus   This observation would appear to be at complete variance with accepted views on passive immunization and we have no explanation to offer   It is possible that there may be an interaction between the immune serum and the dead fixed virus or the live street virus *in vivo* resulting in a ' lag phase ' in immunity production or even temporarily arresting it completely

2   The ' period from infection to death ' was increased in the animals given serum at the beginning of treatment as compared with the remaining animals Details of this observation are given in Table IV —

TABLE IV

Showing the ' average period to death ' in the animals dealt with in Table III according to the method of immunization

Stage of vaccine treatment at which serum was administered	Average period to death in days	REMARKS
Serum given on the first two days of treatment	32 3	Only experiments in which serum was given at the commencement and at the end of vaccine treatment have been included
Serum given on the last two days of treatment	20 9	
Treatment with vaccine alone	21 6	
Controls—No treatment	22 7	

3   In animals receiving serum treatment the period from commencement of symptoms to death is greatly shortened, so much so that in many cases the animals died before typical symptoms of rabies manifested themselves   The diagnosis in all cases was made by the finding of Negri bodies and where these were not found, as was sometimes the case in animals dying without symptoms, the diagnosis was always made by sub-passage to other animals

In connection with the sensitization we have mentioned it may be of interest to detail here an experiment which seems to indicate that this sensitization is especially manifested when the serum is given intravenously   The experiment in question was as follows —

Two groups, each of six rabbits, were given 1 c c of 5 per cent fresh suspension of Paris fixed virus into the flank and the muscles of the neck respectively, followed

immediately by anti-rabic serum given subcutaneously into the flank or intravenously   A third group of six rabbits was given a similar suspension of fresh fixed virus into the flank and muscles of the neck respectively to act as controls   All the rabbits weighed over 1,000 grammes   The results are detailed in Table V —

TABLE V

Showing the sensitizing effect of anti-rabic serum given intravenously

Number of rabbits in each group	Site of administering virus	Mode of administering serum	Died of rabies	Escaped	Average period to death
3	Flank	Subcutaneous		3	
3	Neck	,,	3		13 3
3	Flank	Intravenous	3		13 6
3	Neck	,,	3		10 3
3	Flank	Controls (no serum)	3		13 3
3	Neck	,,	2	1	13 00

A perusal of the table will show that in the case of animals infected in the neck and given anti-rabic serum intravenously the period to death is greatly shortened   It will also be noticed that in the case of animals infected in the flank all those given anti-rabic serum subcutaneously escaped, while all those given the serum intravenously died of rabies

As a general comment on the animal experiments we may say that failure to obtain conclusive, or even suggestive, results is attributable to various factors difficult to control   In the first place the test dose of virulent street virus is usually much more severe than would be the case in persons bitten by rabid dogs and the failure to prevent the onset of rabies in animal experiments does not necessarily imply the inutility of the same methods when applied to human cases bitten by rabid animals   Another difficulty encountered is the great variability in the virulence of different viruses   A very few are so virulent that there may be little or no difference in the results in different groups, while the standard treatment is sufficiently effective to mask differences in the case of many viruses, and to draw conclusions in either event becomes impossible   While, then, we have failed in animal experiments to demonstrate any prophylactic value in

the use of immune serum when used as an adjunct to treatment with carbolized dead virus, a definite advantage seems to have accrued from its use in human cases of bites from rabid animals

(b) Human experiments

This part of the investigation was commenced at about the same time as our animal experiments   It was decided to carry out the tests only on cases definitely at risk in order the more quickly to determine whether the experiments promised to give favourable results in human cases   For this purpose we selected only those cases of class IV (according to Hempt's classification) which were severely bitten This selection virtually meant the creation of a class V, which was, theoretically at least, at even greater risk than class IV   That this selection achieved its purpose is proved by the low death rate in the *remaining* cases of class IV which was only 1 53 per cent (Table VI) as against 2 34 per cent in 1932 which was the lowest percentage ever recorded in any year for class IV cases

For convenience of description we may refer to our selected cases as class V

During the period 24–11–1932 to 30–10–1933 we treated three out of every four class V cases with the standard vaccine treatment plus anti-rabic serum   The fourth case received the standard vaccine treatment alone and served as a control Apart from this there was no selection of cases, which were taken in rotation as they presented themselves for treatment

All cases selected for treatment with serum were given a dose of 20 c c. whole serum subcutaneously on the first and second days of vaccine treatment, the same dose being given to children and adults   The serum seemed to cause little discomfort   The results of the experiment between the dates given above are detailed in Table VI —

TABLE VI

Showing the results of cases of class V treated with and without anti-rabic serum

Description of cases	Number treated	Deaths from hydrophobia	Percentage death rate
Class V cases treated with serum	203	7	3 44
Class V cases treated with vaccine alone (controls)	67	3	4 48
All other class IV cases treated	720	11	1 53

Particulars of the cases which died of hydrophobia are given below —

Cases treated with vaccine plus serum

(1) Patient 6778, Laloo, male, 45 years—severely bitten on face by a rabid jackal, three wounds on bare skin, not cauterized, arrived 15 days late for treatment

(2) Patient 113, Nabbi Ullah, male, 50 years—severely bitten on face by a rabid dog, two wounds on bare skin, not cauterized, arrived 2 days late for treatment

(3) Patient 1267, Narain Das, male, 6 years—severely bitten on face by a rabid dog, twenty-two wounds on bare skin, not cauterized, arrived 2 days late for treatment

(4) Patient 1648, Shiv Kaur, female, 35 years—severely bitten on face, arm, leg and trunk by a rabid leopard, forty-four wounds on bare skin, not cauterized, arrived 3 days late for treatment

(5) Patient 1673, Jagan Nath, male, 12 years—severely bitten on leg by a rabid dog, three wounds on bare skin, not cauterized, arrived 8 days late for treatment

(6) Patient 1849, Ghanshiam, male, 8 years—severely bitten on face by a rabid dog, twelve wounds on bare skin, not cauterized, arrived 7 days late for treatment

(7) Patient 1924, Chet Ram, male, 8 years—severely bitten on face and trunk by a rabid jackal, eleven wounds on bare skin, not cauterized, arrived 2 days late for treatment

Control group treated with vaccine alone

(1) Patient 6878, Khushi Ram, male, 31 years—severely bitten on face and arm by a rabid jackal, four wounds on bare skin, not cauterized, arrived 5 days late for treatment

(2) Patient 7017, Rupa, male, 60 years—severely bitten on face and arm by a rabid jackal, sixteen wounds on bare skin, not cauterized, arrived 2 days late for treatment

(3) Patient 288, S ayera, female, 60 years—severely bitten on face and arm by a rabid dog, three wounds on bare skin, not cauterized, arrived 5 days late for treatment

From the details provided above it is evident that there has been an impartial assignment of cases

The table shows a reduction in the death rate of class V cases from 4 48 per cent to 3 44 per cent This appears to indicate a definite advantage in favour of combined serum and vaccine treatment as against treatment with vaccine alone

To confirm our results and increase the number of observations we now decided to treat *all* cases of class IV without the foundation of a class V within the class IV group, at the same time adhering to the original method of selection, i e , treating three out of every four cases with vaccine plus serum and the fourth with vaccine alone as a control   This part of the investigation is still in progress but the results recorded to date are given in Table VII

TABLE VII

Showing the results of cases of class IV treated with and without anti-rabic serum

Description of treatment	Number treated	Number of deaths from hydrophobia	Percentage death rate
Three out of every four class IV cases treated with serum plus vaccine	381	5	1 31
Controls received vaccine alone	127	4	3 23

The two series are combined in Table VIII in order to compare all cases of classes V and IV treated with and without serum

TABLE VIII.

Showing the results of classes V and IV treated with and without anti-rabic serum

Description of treatment	Number treated	Number of deaths from hydrophobia	Percentage death rate
Three out of every four cases of classes V and IV treated with serum plus vaccine	584	12	2 05
Controls received vaccine alone	194	7	3 60

The results so far obtained are very encouraging and although the figures are still small they appear definitely to point towards the advantage of combining the standard vaccine treatment with the use of anti-rabic serum

3 TREATMENT BY ONE DOSE OF A COMBINATION OF FRESH LIVING FIXED VIRUS AND ANTI-RABIC SERUM

The experiments recorded under this section follow the work of Findlay (1933) on immunization against yellow fever   The ordinary processes of immunization against rabies with killed and attenuated viruses involve comparatively prolonged treatment and it was felt that it would be a great advantage if a method could be devised which would cut down the time of treatment and at the same time be efficacious and perfectly safe   The virus of yellow fever by its conversion into a neurotropic virus has been rendered comparatively innocuous when injected into the subcutaneous tissues and the same can be said to a lesser degree of the rabies virus This innocuousness has been rendered more certain in the case of yellow fever by the combination of yellow fever immune serum with the live virus in the one dose method of vaccination of human beings   It was with the idea of seeing whether a similar line of immunization would be successful against rabies that the experiments detailed below were carried out —

Experiment No 77 —One hundred and sixty-two monkeys were used in the experiment and divided into six groups of twenty-seven each   These groups were immunized as follows ·—

> *Group A* —With one dose of unconcentrated anti-rabic serum plus fresh live fixed virus
>
> *Group B* —With one dose of concentrated serum (combined eu-globulin and pseudo-globulin) plus fresh live fixed virus
>
> *Group C* —With one dose of concentrated serum (eu-globulin) plus fresh live fixed virus
>
> *Group D* —With one dose of concentrated serum (pseudo-globulin) plus fresh live fixed virus
>
> *Group E* —With one dose of fresh live fixed virus
>
> *Group F* —Controls—No immunization

In the four groups A, B, C and D the dose of whole serum or its concentrates was 1 c c and the dose of fresh live fixed virus was 0 5 c c of a 10 per cent emulsion of sheep's brain   Group E received 0 5 c c of the 10 per cent emulsion alone

The immune serum and the concentrates were infiltrated intradermally round a circular area 1½ to 2 inches in diameter on the flank of each animal   Two to four hours later the injection of fresh live fixed virus was given subcutaneously towards the centre of the infiltrated area from one side

Twenty-one days later all the six groups were infected in the muscles of the neck with 2 c c of a 2 per cent emulsion of street virus from a first passage sheep   The results are given in Table IX —

TABLE IX

Showing the results of immunization with a single intradermal injection of fresh live fixed virus combined with anti-rabic serum

Nature of immunization	Original number of monkeys	Died prior to infection	Died of other causes after infection	Died of rabies	Percentage of deaths due to rabies (excluding deaths from other causes)	Remarks
1 c c unconcentrated serum intradermally plus 0 5 c c of 10 per cent live fixed virus subcutaneously	27			1	3 70	
1 c c combined globulins intradermally plus 0 5 c c of 10 per cent live fixed virus subcutaneously	27		1	2	7 70	
1 c c eu globulin intradermally plus 0 5 c c of 10 per cent live fixed virus subcutaneously	27	1		2	7 70	26 animals infected
1 c c pseudo globulin intradermally plus 0 5 c c of 10 per cent live fixed virus subcutaneously	27	1	3	2	8 70	26 animals infected
0 5 c c of 10 per cent live fixed virus subcutaneously	27	3		1	4 17	24 animals infected
Controls—No treatment	27			16	59 26	

The results of this experiment as set forth in the table give no indication of any advantage in the use of anti-rabic serum in addition to live virus as against live virus alone   All the methods of single dose vaccination, however, show a considerable saving effect but the experiment was somewhat unsatisfactory owing to the comparative mildness of a virus which only killed 59 26 per cent of controls   On the other hand it is possible that such mild viruses in the doses used in our experiments give a truer approximation to the risk at which patients bitten by rabid animals are, under ordinary conditions, than the use of potent viruses giving a death rate of 90 per cent in controls

A second experiment on similar lines but on a smaller scale was subsequently performed and the details are given in Table X —

TABLE X

Showing the results of immunization with a single intradermal injection of fresh live fixed virus combined with anti-rabic serum

Group	Original number of monkeys	Died prior to infection	Died of other causes after infection	Died of rabies	Percentage of deaths due to rabies (excluding deaths from other causes)	REMARKS
A	15	2		10	77 00	13 animals infected
B	15	1	1	6	46 15	14 ,, ,,
C	15	1		4	28 57	14 ,, ,,
D	15	2		6	46 15	13 ,, ,,
E	15	2	1	4	30 77	13 ,, ,,
Controls	15			10	66 67	15 ,, ,,

A=Treated with unconcentrated serum   D=Treated with psendo globulin fraction
B=Treated with combined globulin fractions   E=Treated with fresh live fixed virus only
C=Treated with eu globulin fraction   F=Controls—Not treated

In this experiment the virus is a more potent one and the saving effect of the vaccination is less

In Table XI the results of these two experiments are combined —

TABLE XI.

Showing the results of immunization with a single intradermal injection of fresh live fixed virus combined with anti-rabic serum (combination of two experiments)

Nature of immunization	Original number of monkeys	Died prior to infection	Died of other causes after infection	Died of rabies	Percentage of deaths due to rabies (excluding deaths from other causes)	Remarks
1 cc unconcentrated serum intradermally plus 0 5 cc of 10 per cent live fixed virus subcutaneously	42	2		11	27 50	40 animals infected
1 cc combined globulins intradermally plus 0 5 cc of 10 per cent live fixed virus subcutaneously	42	1	2	8	20 51	41 " "
1 cc eu-globulin intradermally plus 0 5 cc of 10 per cent live fixed virus subcutaneously	42	2		6	15 00	40 "
1 cc pseudo-globulin intradermally plus 0 5 cc of 10 per cent live fixed virus subcutaneously	42	3	3	8	22 22	39 "
0 5 cc of 10 per cent live fixed virus subcutaneously	42	5	1	5	14 17	37 "
Controls—No treatment	42			26	61 90	

The general result of the two preceding experiments is to show that one inoculation of a vaccine containing fresh live virus has a considerable saving effect   The influence of the serum or its fractions on the results is not manifest

4   THE USE OF FRESH LIVING FIXED VIRUS AS AN ADJUNCT TO TREATMENT BY CARBOLIZED DEAD VIRUS

It is a commonplace of immunology that in very few cases, if ever, does a dead vaccine confer as high a degree of immunity as an infection, even a mild one, with the living organism, of the same species   It seems reasonable, therefore, by analogy to infer that immunization with the live virus of rabies will confer a higher immunity than when only a dead vaccine is used   The relative innocuousness of fixed virus in the subcutaneous tissues and its capacity to produce immunity against street virus enable the principle of immunization with a live virus to be applied As hydrophobia, however, whether induced by fixed or street virus, is a disease from which there is no recovery, it is essential that some degree of immunity should already be present before even fixed live virus is inoculated into the tissues   In applying the principle, therefore, of using fresh live fixed virus to produce a solid immunity we have ensured the presence of a degree of preliminary immunity by preceding the use of live virus by a dead vaccine   The results of this policy exceeded our anticipations

Experiment

A completely paralysed sheep infected with Paris fixed virus was killed on the ninth day after infection and its brain removed   This was emulsified into an 8 per cent suspension in normal saline to which 1 per cent of pure carbolic acid was added   The suspension so made was filtered through muslin, incubated at 37°C for twenty-four hours and then diluted with normal saline to make a 6 per cent vaccine containing 0 75 per cent of carbolic acid

Twenty-four dogs were weighed and placed in two groups   Group A consisted of dogs weighing twenty pounds and over   Group B consisted of dogs weighing under twenty pounds

The dogs of group A were given 10 c c of the 6 per cent vaccine daily for 7 days and those of group B, 5 c c of the vaccine for the same period

As we wished to make a test of the innocuousness of the 6 per cent vaccine as regards local reactions as well as its efficiency in combination with live virus we made a deliberate selection of sickly and emaciated dogs and to this must be attributed the considerable mortality during the course of the experiment

Three weeks after completion of the course of immunization the thirteen surviving animals were each given 5 c c of a 1 per cent suspension in normal saline solution of fresh Paris fixed virus sheep's brain subcutaneously, followed, after a further week's interval, by 10 c c of a similar suspension

Ten days later all the animals were given an infecting dose of 2 c c of a 1 per cent suspension of first passage street virus deep into the muscles of the neck, half the dose being given on each side   Eight control dogs were similarly infected   The details of the results of this experiment are given in Table XII —

TABLE XII

Showing the results of immunization with dead vaccine followed by fresh live fixed virus

Dog number	Sex	Group	6 per cent vaccine from 24-2-1933 to 2-3-1933 daily	1 per cent fresh fixed virus on 23-3-1933	1 per cent fresh fixed virus on 30-3-1933	1 per cent street virus on 9-4-1933	Date Died of rabies	Date Died of other causes	RESULTS
			c c	c c	c c	c c			
2	M	A	10	5	10	2		13-7-33	Passage, negative
3	F	A	10	5	10	2			
5	F	A	10	5	10	2			
6	F	A	10	5	10	2			.
7	M	A	10	5	10	2		2-7-33	Passage, negative
8	M	A	10	5	10	2			
9	M	A	10	5	10	2			
10	M	A	10	5	10	2			
11	F	A	10	5	10	2			.
14	F	B	5	5	10	2		3-8-33	Passage, negative
16	F	B	5	5	10	2			
19	F	B	5	5	10	2		16-5-33	Passage, negative
22	F	B	5	5	10	2		2-6-33	,, ,,
25	F	Control				2	26-4-33		Negri bodies, positive
26	F	,,				2	3-5-33		,, ,,
27	M	,,				2	26-4-33		,, ,,
28	M	,,				2	28-4-33		,, ,,
29	M	,,				2	22-4-33		,, ,,
30	F	,,				2	22-4-33		,, ,,
31	M	,,				2	25-4-33		,, ,,
32	F	,,				2	24-4-33		,, negative*.

* First rabbit passaged on 1-5-33, died 4-5-33   Second rabbit passaged on 4-5-33, died on 8-5-33

A perusal of the table will show that the results were highly satisfactory, cent per cent of the immunized animals being protected, while cent per cent of the controls died of rabies   This result was the more noteworthy because we consider dogs to be naturally more susceptible to rabies than monkeys on which most of our experiments have hitherto been conducted, because even when employing the latter we have never been able closely to approach a cent per cent saving in experiments with dead vaccine   Of the thirteen immunized animals five died of other causes more than two months after infection, chiefly on account of their original condition as explained above   The remaining eight animals were alive five months later The brains of all animals dying of other causes were examined for Negri bodies and rabbits were sub-passaged   In none were Negri bodies found and all the passage rabbits remained healthy

Of the eight control dogs seven died of rabies within twenty-four days of infection   The brains of all were examined and Negri bodies were found in all   The eighth dog showed definite symptoms of rabies and died fifteen days after infection This animal unfortunately died during the early part of the night in the hot weather and by the morning its brain was soft and decomposed   Two rabbits subpassaged with the brain died of septicæmia within four days owing to the condition of the dog's brain

As the results recorded above are better than any we have ever obtained by the use of dead vaccines alone the difference must be attributed to the addition of fresh living fixed virus to the immunizing treatment

Summary and Conclusions

(1)   The method of preparation, standardization and concentration of antirabic serum has been described

(2)   The use of anti-rabic serum as an adjunct to treatment with carbolized dead virus has been described and it is considered to mark an advance on the present methods of treatment by vaccine alone

(3)   Treatment by one dose of a combination of fresh living fixed virus and anti-rabic serum has been shown to have a considerable immunizing value although the main effect appears to have been exerted by the live virus, the serum being used mainly to render the use of the live virus more safe

(4)   The use of fresh living fixed virus as an adjunct to treatment with carbolized dead virus has been shown to be the most effective method yet tried for the immunization of dogs and, therefore, probably of other animals also

(5)   It is considered that future lines of advance towards the most efficacious means of producing a solid immunity against rabies will be along the path of utilization of anti-rabic serum and fresh live fixed virus, possibly combined with the use of a dead vaccine

Acknowledgments

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NOTE ON RABIES FIXED VIRUS AS AN ANTIGENIC AGENT WHEN INACTIVATED BY THE PHOTODYNAMIC ACTION OF METHYLENE BLUE

BY

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IN a previous communication (Shortt and Brooks, 1934) we have described the inactivation of rabies fixed virus by the photodynamic action of methylene blue and, at the end of our account, mentioned the possibilities opened up for the use of this action in the preparation of an anti-rabic vaccine

With a view to testing these possibilities we have carried out a few preliminary experiments but had no intention of publishing the results of these until more exhaustive tests had been carried out   The fact, however, that certain work on similar lines by Galloway (1934) has already been published and gave indications of the value of a vaccine prepared from rabies virus inactivated by the photodynamic action of methylene blue, has induced us to record our results, incomplete as they yet are, because they lead to an entirely contrary conclusion   This question will be discussed after the results of our experiments have been given

Two experiments were performed by us the details of which are set forth below —

EXPERIMENT 72 (A)

Twenty-four rabbits were divided into five groups which were treated as indicated below —

Group A —Six rabbits, each of which was given 10 c c of a 1 per cent rabies fixed virus suspension in 1 in 20,000 solution of methylene blue which had been exposed to sunlight for 20 minutes ; The inoculum was given subcutaneously into four places in the abdomen

> *Group B* —Six rabbits, treated similarly to group A except that the inoculum was 20 c c of the blue-virus suspension
>
> *Group C* —Three rabbits, each of which was given 10 c c of a 1 per cent rabies fixed virus suspension in distilled water which had been exposed to sunlight for 20 minutes
>
> *Group D* —Three rabbits, treated similarly to group C except that the inoculum was 20 c c of the ' distilled water-virus ' suspension
>
> *Group E* —Six rabbits, untreated to act as controls of the virulence of the virus

Twenty-two days after immunization all the animals were infected with street virus, the dose being 1 c c of a 1 per cent suspension of street virus given into the muscles of the neck

The results of this experiment are given in Table I —

TABLE I

Showing the results of treatment with vaccine inactivated by the photodynamic action of methylene blue compared with those obtained by the use of vaccine not so inactivated

Group	Number of rabbits	Dose of blue-virus or virus only in c c	Died of rabies	Died of other causes	Survived	Percentage of deaths due to rabies (excluding deaths from other causes)
A	6	10	6			100
B	6	20	5		1	83 3
C	3	10	1		2	33 3
D	3	20			3	*Nil*
E	6		4	2*		100

* Died on the 2nd and 3rd days after infection

EXPERIMENT 72 (B)

In this experiment the antigenic value of the vaccine inactivated by the photodynamic action of methylene blue was compared with that of the 5 per cent carbolized vaccine in ordinary use at Kasauli   Ninety rabbits were divided into three groups which were treated as indicated below —

> *Group A* —Thirty rabbits, each given a daily dose of 1 c c of 5 per cent carbolized vaccine for fourteen days (equivalent to 70 c c of a 1 per cent suspension)

Group B —Thirty rabbits   On the 1st, 8th and 14th days of treatment of animals in group A each animal of group B was given 12 c c , 12 c c and 11 c c of a 1 per cent suspension of rabies fixed virus in 1 in 20,000 methylene blue which had been exposed to sunlight for 20 minutes These animals received exactly half the quantity of brain substance given to animals in group A

Group C —Thirty rabbits, untreated to act as controls of the virulence of the virus

Nineteen days after immunization all the animals were infected with street virus the dose being 1 c c of a 1 per cent suspension of street virus given into the muscles of the neck   The results of this experiment are given in Table II —

TABLE II

Showing the results of treatment with vaccine inactivated by the photodynamic action of methylene blue compared with those obtained by the use of the 5 per cent carbolized vaccine in ordinary use at Kasauli

Group	Number of rabbits	Died of rabies	Died of other causes	Survived	Percentage of deaths from rabies (excluding deaths from other causes)
A	30	4	11	15	21 05
B	30	13	5	12	52 00
C	30	22	5	3	88 00

DISCUSSION

The general conclusions to be drawn from these experiments clearly indicate that the photodynamic action of methylene blue greatly impairs the antigenic value of fixed rabies virus   The contrary results obtained by Galloway may possibly be explained in two ways   In the first place after the preliminary immunization with the methylene blue-inactivated vaccine the infecting agent used by him was fixed virus, i e , the same agent as that used in the vaccine, whereas we used street virus as the infecting agent since any practical application of the vaccine would be against the infecting agent in nature, viz , street virus

In the second place Galloway, as his source of light, used a 300 c p filament lamp, whereas we used sunlight, a much more powerful source of light   We think it possible, therefore, that the virus in his vaccine may not have been completely inactivated and that the saving effect was actually due to the surviving live virus in the vaccine   The possibility of his ' inactivated ' suspension containing live virus will be evident when it is considered that with the source of light used by him he was unable to inactivate unfiltered suspensions of virus which had been centrifuged for 10 minutes at 2,500 revolutions per minute and subsequently

sedimented for 24 hours while with sunlight we were able to inactivate a 1 per cent suspension which had only been centrifuged for 2 minutes in a hand-driven centrifuge and not subsequently sedimented[*]

Against this view is the fact that Galloway controlled his results with rabbits into which the 'inactivated' virus was inoculated intra-cerebrally without producing infection. This is strong evidence of the complete inactivation of the virus but does not rule out the possibility that an attenuated virus, capable of producing immunity but incapable of giving rise to infection before it was dealt with by the rabbits' defence mechanism, may have been present in the mixtures used for immunization Moreover, if the rabbits inoculated subdurally with this mixture were operated on in daylight it is quite possible that the exposure of the extremely attenuated suspensions to daylight may have completed their inactivation before they were inoculated The inference from such a supposition would appear to be that if the photodynamic action of methylene blue is to be of any use in the preparation of vaccines its action must stop short of complete inactivation in the sense of a 'dead' virus

CONCLUSION

The photodynamic action of methylene blue on rabies fixed virus greatly impairs its value as an antigenic agent and the application of the principle of photodynamic action of methylene blue is not likely to be of practical value in the preparation of an anti-rabic vaccine

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* In the actual experiments recorded above the blue virus suspension was not centrifuged

STUDIES ON THE PROTEIN FRACTIONS OF BLOOD SERA

Part II

BLOOD SERA OF OPIUM ADDICTS

BY

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It is now well recognized that the effect of morphine addiction is not only psychological but it is definitely pathological so far as the system of the addicts is concerned. The appearance of constipation on addiction and of diarrhœa on withdrawal after a short addiction are all popularly known (cf. Barbour, Hunter and Richey, 1929). Maguin (1909) observed that diminished thirst and oliguria are the common accompaniments of morphine addiction and that polyuria commences on withdrawal. Rowntree (1922) reported a disturbance in the water balance in the system of morphine addicts. Pierce and Plant (1928) observed a dilution of blood amounting to as much as ten per cent on addiction. From a study of the specific gravity of the whole blood Barbour, Hunter and Richey (*loc. cit.*) have also made similar observations. The immoderate return of the suppressed secretions on withholding morphine from addicted men also bears out the above observations. The outstanding features as observed by Maguin (*loc. cit.*), Sollier (1910) and others in the last mentioned case are the excessive sweating, salivation and diarrhœa.

In addition to these effects morphine is known to increase the carbon dioxide tension of blood (Cobet, 1923; Henderson and Haggard, 1916).

These effects have a far reaching influence upon the physiology and also on the various physico-chemical processes going on in the human body. The depression of the respiratory centre with the consequent alteration in the carbon dioxide tension is likely to bring about a change in the alkaline reserve and also in the serum reaction. The disturbance in the water balance of the system, on the other hand, may also bring about a change in the osmotic pressure of the serum as well as its constituent protein fractions.

The present work was undertaken to study the changes in the physico-chemical properties and also the proteins of the blood sera in such cases. Since in India cases of morphine addiction are not very common but people, owing to its easy availability and a comparatively lower price, take to raw opium as produced in this country, our investigations were concerned only with the opium addicts as commonly found here. The physico-chemical properties, such as the surface tension, the relative viscosity, the pH and the buffer action as well as the various protein fractions, have been determined in the blood sera of these addicts from the point of view of their pathology and treatment. In the present paper the observations were carried on only on the opium eaters. The studies into the blood sera of the opium smokers and of other addicts will form the subject matter of separate communications.

Experimental procedure

Ten c.c. of venous blood were drawn from each addict into a clean and sterilized dry test-tube from which approximately 5 c.c. of serum were obtained. In all the cases the addicts were intimately known and well diagnosed to be free from other diseases. It will not be out of place to mention here that it is very difficult to find such addicts and to persuade them to part with their blood because of their innate nervousness.

In all cases the blood was drawn within 2 to 3 hours after they had taken their daily dose.

The methods used to determine the physical properties as well as the protein fractions are the same as described in Part I of this series (Chopra, Mukherjee and Rao, 1934). Proteins were determined at 17·5°C and the amount precipitated by 33 per cent saturation with ammonium sulphate was regarded as the euglobulin fraction.

All the experiments were done with scrupulous cleanliness and care. The test-tubes, pipettes, etc., were all cleaned with potassium dichromate-sulphuric acid mixture and dried after experiment with each serum. The mean of the three readings was taken in each case. The results obtained have been tabulated below.

Results and discussion

The data obtained from a study of the blood sera of the opium addicts have been given in Table I. For comparison with similar figures for normal Indian subjects Tables II and III of Part I of this series are referred to (Chopra, Mukherjee and Rao, *loc. cit.*)

(1) *Hydrogen-ion concentration*

From Table I it is evident that pH in all cases is near the lower limit of the normal range and the average figure, although within the normal range, is a little lower than that found for normal Indian subjects   Our observation on the lowering of pH is in accordance with those of Cobet (*loc cit*) and Henderson and Haggard (*loc cit*), who have shown that morphine increases the carbon dioxide tension and lowers the pH   According to these authors the effect of morphine is to produce an uncompensated primary carbon dioxide excess in the blood, while Barbour, Hunter and Richey (*loc cit*) (also Peters and van Slyke, 1931) have found that the alkaline reserve is diminished in these cases   These two factors, viz , the increase of free primary carbon dioxide, an outcome of the increase in the carbon dioxide tension (according to Henry's law) and the diminution of the alkaline reserve or the bicarbonate content of the blood both act in the same direction in lowering the pH, as will be evident from a consideration of the Henderson-Hasselbalch equation *

From the physiological point of view such changes in the pH are evident   The respiratory centre is the organ that regulates the pH of the blood by eliminating the accumulated carbon dioxide   The increase in the carbon dioxide tension is known to decrease the pH or increase the hydrogen-ion concentration of the blood which, probably by diffusion or otherwise, affects the hydrogen-ion concentration at the respiratory centre and thereby stimulates it, with the result that the excess of carbon dioxide, which is responsible for this increase in the hydrogen-ion concentration, is eliminated and the pH comes back to its normal value   The lower pH values of the opium addicts' blood sera indicate a loss of sensitiveness on the part of the respiratory centre   This depression may be due to some selective action of opium on such nerve-cells or more probably due to a general depression of all the nervous tissues consequent upon the drainage of lecithin from these to form an increased amount of euglobulin observed in such cases (*vide infra*) or due to some change in the adsorption of ions by the nerve-cells owing to a change in the interfacial tension and also in the dielectric constant of the medium brought about by the opium alkaloid in the blood

(2) *Buffer action*

Buffer action is considerably diminished in all the cases   This diminution may be partly due to a fall in the percentage of the proteins and partly to the decrease of the alkaline reserve (or the bicarbonate content) of the blood observed in such cases (cf Barbour, Hunter and Richey, *loc cit*)   The observations of Henderson and Haggard (*loc cit*) on the increase of carbon dioxide tension induced by morphine in the system, moreover, give a further clue to such diminution of the buffering properties   It is well known that in the serum the main buffering

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* The relationship between the pH, the carbonic acid concentration and the bicarbonate content of blood is given by Henderson Hasselbalch equation —

$$pH = pK + \log \frac{(BHCO_3)}{(H CO_3)}$$ where $pK = - \log K$, the apparent dissociation constant of carbonic acid,

(BHCO$_3$) and (H$_2$CO$_3$) are the concentrations of the bicarbonate and the carbonic acid in the blood respectively

TABLE I.

Opium addicts' blood sera

Number	Daily dose (grams)	Surface tension in dynes	Relative viscosity	pH	Buffer action (0 4 c c serum and 0 6 c c 0 01 N HCl)	Albumin*	Pseudo-globulin*	Euglobulin*	Total proteins*
1		62 8	1 50	7 44	6 69				
2	.			7 39					
3	.	.		7 49					
4	4 5	63 3	1 55	7 42	6 74				
5	2	62 6	1 44	7 42	6 76				6 60
6	4 5	59 5	1 57	7 48	6 70				6 82
7	22 5	59 9	1 33	7 57	6 39				5 27
8	25	64 4	1 32	7 45	6 66				5 16
9	25	61 6	1 50	7 34	6 54	4 71	1 38	0 29	6 38
10	20	56 6	1 43	7 48	6 40	5 32	1 90	0 30	7 52
11	20	57 7	1 30	7 23	6 28	4 91	1 65	0 35	6 91
12		67 6	1 25	7 27	6 44	4 03	0 91	0 34	5 28
13	2	59 0	1 40	7 25	6 46	4 91	2 30	0 34	7 55
14	5	57 7	1 46	7 39	6 68	4 29	2 84	0 35	7 48

15	25	60 0	1 44	7 38	6 36	4 77	0 60	0 72	6 09
16	5	61 6	1 44	7 37	6 30	5 10	0 90	0 45	6 45
17	10–12	57 8	1 33	7 20	6 18	4 85	1 21	0 39	6 45
18	20	59 1	1 50	7 28	6 34	5 50	1 20	0 48	7 18
19	2	60 6	1 30	7 29	6 51	4 66	1 40	0 46	6 52
20	4	59 3	1 41	7 27	6 44	5 50	1 26	0 43	7 19
21	5	63 6	1 50	7 21	6 01	5 85	1 12	0 31	7 28
22	3	60 3	1 20	7 33	6 31	4 90	1 20	0 42	6 52
23	3	61 1	1 35	7 23	6 35	5 00	1 25	0 45	6 70
24		62 3	1 41	7 21	6 28	4 80	1 27	0 38	6 45
25		61 7	1 42	7 24	6 19	4 89	1 21	0 43	6 52
Average		61 3	1 41	7 30	6 43	4 94	1 40	0 40	6 60
Average for normal Indians		60 1	1 60	7 48	6 88	4 61	2 65	0 18	7 44

(Cf Chopra, Mukherjee and Rao, loc cit , Tables II and III)

* 'Albumin', 'Pseudo globulin', 'Euglobulin' and 'Total proteins' stand for the number of grammes of these substances in 100 c c of serum

properties are due to the bicarbonate alkali present there   'The availability of this bicarbonate alkali of the blood depends upon the ability of the organism to eliminate the carbon dioxide set free when the bicarbonate is decomposed by other acids When the carbonic acid itself collects in the blood the bicarbonate becomes relatively unimportant as a buffer ' (Peters and van Slyke, *loc cit*)   In the case of opium addicts a slight depression in the respiratory centre has been well recognized for a long time, as a result of which the carbon dioxide elimination does not take place as effectively as in the case of normal individuals   Hence the bicarbonate alkali, which is already diminished in such cases, is further rendered inefficient by the presence of excess of carbon dioxide, owing to which a lower buffer action is to be expected

(3) *Surface tension and relative viscosity*

The increase in the surface tension and the decrease in relative viscosity of sera both point to the fact that the sera contain relatively less proteins and more fluid The relative viscosity of the whole blood is found to increase in some cases as compared with such figures for normal subjects (*vide* Table II), while in others the deviation from the normal is not very marked   This increase in the viscosity of the whole blood can also be explained in terms of the carbon dioxide tension   When the carbon dioxide enters the blood it diffuses both into the plasma and into the corpuscles   The corpuscles have some mineral bases in them, a part of which is associated with the proteins present therein   Carbon dioxide deprives the proteins of their bases and forms bicarbonate with them   So there is an increase in the concentration of the bicarbonate in the corpuscles as a result of which the osmotic pressure inside the corpuscles increases   More water is, therefore, drawn in and the cells swell to a considerable extent (*vide* McClendon and Medes, 1925)   This swelling of the cells renders the whole blood more viscous   Hence from these considerations, the viscosity of the whole blood of the opium addicts is the resultant of two effects   (1) the swelling of the red cells which increases viscosity and (2) a lowering of viscosity in the serum   The net effect of these will give the resulting viscosity of the whole blood   It may either be greater than, equal to, or less than that of the normal blood according as one or other of these two factors predominates

Table II

Relative viscosity

Number	Normal subjects		Opium addicts	
	Whole blood	Serum	Whole blood	Serum
1	3 12	1 60	3 20	1 41
2	3 05	1 57	3 06	1 53
3	3 15	1 63	3 25	1 37
4	3 09	1 65	3 13	1 43

The rise in viscosity of the whole blood and the lowering observed in the serum seem to be very interesting since they may be looked upon as an effort on the part of the system to keep up the viscosity equilibrium of the blood to facilitate the blood circulation   The increase in viscosity due to the swelling of the red cells is, as it were, being balanced by a decrease in the serum in some of the cases

(4) *Total proteins and protein fractions*

The total proteins were found to be decreased to a slight extent in all the cases studied   The determination of the individual protein fractions reveals a relative lowering of the total globulins and an increase in the albumin fraction in some of the cases   In no case is albumin found to diminish below the values found for normal Indian subjects   But though the total globulins are diminished in these cases, the euglobulin fraction is found to increase

These protein changes are of considerable interest from the point of view of the water balance of the system which has been observed by many authors (*vide supra*) to be disturbed   The principal function of the proteins in the body is to maintain the physical and the physico-chemical state of the blood (Peters and van Slyke, *loc cit*)   This rôle, played chiefly by the albumin and the globulin fractions, may be ascribed to their osmotic attraction for water maintaining the fluid balance between the blood, the intercellular tissue spaces and the serous cavities (cf Starling, 1895) *

In the present case the total proteins are found to be slightly diminished   The diminution may be due either to an actual diminution in the proteins themselves in the blood sera or to an increase in the fluid content of the sera or to both   But judging from the previous work done   along this direction in this laboratory as well as from the various clinical and physico-chemical findings in these cases made by different observers (*vide supra*), we are led to believe that there may be a real increase in the fluid content of the sera in these cases

Assuming, however, that there is an increase in the fluid portion in such sera, as we believe it to be, we are led to conclude that the albumin and the euglobulin portions must have increased to a considerable amount before the fluid went in to dilute the blood and to reduce their concentration to what they are   The explanation of such an inflow of the fluid into the blood is obvious because the increase in the albumin fraction is known to increase the oncotic pressure (Govaerts, 1923) of the serum   From the picture of the fluid exchange in the capillaries as presented by Schade, Claussen *et al* (1926) it is quite clear why there should be more inflow of the tissue fluids into the blood especially in view of the fact that in such cases the osmotic (oncotic) pressure increases owing to a relative increase in

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* The serum proteins in diffusion equilibrium with relatively protein free saline solutions in tissue spaces attracts water for two reasons   (1) The molal concentration of the non diffusible proteins in the plasma and (2) an unequal distribution of the diffusible ions, chiefly $Na+$, $Cl-$, and HCO_3-, between the plasma and the fluids in the tissue spaces

Govaerts (1923) and Schade and Claussen (1924) termed this osmotic pressure due to the serum colloids the 'oncotic pressure' and found its value to be intermediate between the arterial and the venous blood pressures   Govaerts (1925 1926) from simultaneous determinations of the albumin, globulin and the osmotic pressure of sera in a number of human subjects showed that the oncotic pressure exerted by albumin is nearly four times as great as that due to the globulins

albumin   We, therefore, conclude from these considerations as well as from the various findings in this laboratory and elsewhere that opium addiction increases the albumin of the blood sera as a result of which the osmotic (oncotic) pressure also increases and more fluid from the tissues is drawn into the blood   This process is promoted by the swelling of the red cells due to osmotic attraction of the fluid inside As a result of this increased oncotic pressure and the change in the hydrostatic pressure of the inflow of the blood into the capillary due to a change in the viscosity of the whole blood as observed in some of the cases, more fluid from the tissues is drawn into the blood   It may be suggested herewith that this rise in the albumin contents of the sera may account for the decreased albuminuria by opium administration as observed in some of the nephritis and other cases (*vide* Bose, 1931)

The large decrease in the pseudo-globulin contents may, however, be due to two reasons   (1) the actual dilution of the serum, and (2) the probable denaturing influence of carbon dioxide on the globulin fractions   This seems to be possible from the work of Hardy and Gardner (1910) from which it appears that the pseudo-globulin is the only form of globulin present in the blood of living animals

As regards the euglobulin fraction, we find that there is a small increase in all the cases studied   Euglobulin differs from pseudo-globulin since it contains phosphorus, and Chick (1924) has put forward evidence to show that this is due to the association of lecithin with the proteins in the form of an adsorption compound and Chick considers that it is derived from the pseudo-globulins by the denaturing influence of lecithin   From this point of view it is quite likely that an increase in the euglobulin fraction must have taken place by a greater adsorption of lecithin by the pseudo-globulins   This again might have been probably promoted by changes in the dielectric constant brought about by opium in the blood   Hence we might expect a disturbance in the equilibrium of lecithin in the system   The greater adsorption of lecithin to form increased amount of euglobulin, decreases the lecithin in the blood proper, which again is possibly being replenished by drawing upon the lecithin of the nerve-cells both central and peripheral   This picture of the lecithin outflow from the nervous tissues, on the basis of Chick's work, to produce an increased amount of euglobulin may account for the changes in the nervous mechanism of the addicts observed by Chopra *et al* (unpublished)   The work of Ma (1932) has thrown considerable light upon this   From a cytopathological study he found that the Golgi apparatus in the tissue-cells of the addicts seemed to be damaged or altered from the normal in as much as, unlike the normal subjects' cells, these do not take stains with neutral red   The administration of lecithin which has been found by him to cure the withdrawal symptoms enables these cells to take this stain and to come back to their normal conditions

From the above considerations it appears to be very suggestive that the drainage of these glycero-phosphates from the nerve-cells may be overcome to a certain extent by the administration of diets rich in such things which may consequently combat the physical changes in the sera observed by various workers as well as by ourselves   This may also help in restoring the normal balance of lecithin in the blood and thus indirectly help in overcoming the dreaded withdrawal symptoms which may possibly be due to the injury of the nerve-cells following the deprivation of the lecithins   It is also indicative of the fact that in addition to the diets rich in these phosphates the administration of drugs like lecithins may be of

immense value in the treatment of opium addiction and in overcoming the weakness and the nervous breakdown in the addicts as the outcome of the drug habit

These inferences have been to a certain extent corroborated by our observations in the field   The rich and the well-off persons who, although taking moderate doses of opium for prolonged periods take plenty of milk and a diet rich in phosphates, etc , do not suffer from nervous breakdown and loss of weight like those who can ill afford to do that   The Sikhs and the Rajputs who live a country life show very little nervous and physical changes   This may be due to their diet which consists mainly of milk, wheat, meat and eggs   These races have been taking opium for centuries and one can hardly trace any degeneration amongst them, which may be accounted for as due to the opium habit   Addicts from Assam, Bengal and the Central Provinces, on the other hand, whose diet is chiefly composed of rice and vegetables have been observed to suffer more than the above races and signs of mental and physical degeneration are clearly visible (cf Chopra and Chopra, unpublished)

The above conclusions are also borne out from a study of the health of the addicts of two types of people of the same district   Nowgong district in Assam consists of the plains and the Mikir hills, the latter inhabited by the Mikirs who consume more opium per head per annum (14 tolas) as compared with those living in the plains who consume 9 7 tolas per head per annum *(vide* Excise Administration Report, Assam, 1933)   Although the climate of the hilly region is found to be worse than that of the plains in this part of the country, the health of the Mikirs is much better than the Assamese of the plains, due most probably to the fact that the former take milk and meat in quantities which the latter can not afford to do

Hence our conclusions regarding the drainage of lecithin from the nerve-cells seem to be justified in a way   But the administration of lecithin as a curative drug in cases of addiction and withdrawal has been the subject matter for further investigation in this laboratory and our results will be published in a future communication

SUMMARY AND CONCLUSIONS

1   The physical properties of the blood sera of opium addicts have been studied with the following results —

The pH is lowered as compared with that of normal individuals   This corroborates an increase in the carbon dioxide tension and a consequent loss of sensitiveness on the part of the respiratory centre of the addicts

Buffer action is considerably diminished, probably due to a lower alkaline reserve observed in such cases and also to the fact that this lower alkaline reserve is further rendered inefficient as a buffer, owing to the increased carbon dioxide tension

The increase in the surface tension points to a relative increase in the fluid content of the sera and a diminution in the protein contents

The decrease in the relative viscosity also corroborates the above conclusions The viscosity of the whole blood is found to increase in some of the cases, most probably due to the swelling of the red blood cells on account of the increased carbon dioxide tension

2   The total and the individual proteins have also changed in the following way —

The total proteins are diminished   The albumin fraction is not diminished in any case, on the contrary, it is found to increase appreciably in many of the cases This increase in the albumin fraction is probably responsible for the increase in the fluid content of these sera as observed by various workers

The pseudo-globulin fraction is considerably diminished   The euglobulin fraction is found to increase   From Chick's (*loc cit*) observations that euglobulin is an adsorption compound of pseudo-globulin with lecithin, the drainage of lecithin from the nerve-cells seems to be probable, and an explanation for the degeneration of the nervous tissues from this point of view has been indicated

A mode of treatment has also been suggested herein for both addiction and withdrawal

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STUDIES ON THE PROTEIN FRACTIONS OF BLOOD SERA

Part III

MALARIAL SERA DURING AND AFTER THE RIGOR STAGE

BY

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IT is a well-known fact that in many pathological conditions the physical properties of blood sera as well as the constituent proteins thereof undergo definite changes (Basilico, 1926 , Rohrer, 1922 , Schretter, 1926 , Ganslen and Maier, 1927 , Achelis, 1926 , Chopra and Chaudhury, 1928, 1929) In many cases again, for instance in kala-azar (Napier, 1921 , Brahmachari, 1923 , Roy, 1924 , Chopra, Chaudhury and De, 1931), such changes have got not only a well-defined diagnostic value but also a prognostic significance

In chronic cases of malaria the changes in the different protein fractions have been studied by Lloyd and Paul (1929) and their gradual return to the normal values along with the quinine treatment has also been observed Very little work has, however, been done on the changes occurring in connection with the rigor state which is one of the chief characteristics of malarial fever and it is not known how the different constituents of the blood are affected during these paroxysms Such studies were carried out by us and this paper embodies the results of these observations

The patients infected with malaria were admitted under the senior author (R. N C) into the Carmichael Hospital for Tropical Diseases   In order to identify the species of the infecting parasites correctly the anti-malarial treatment was withheld and daily examinations of the blood were carried on *for two or three days* , in doubtful cases cultural examinations were also done   Ten c c of the blood were then taken from these patients, with the usual precautions, during the stage of rigor and kept in a cool chamber to allow the serum to separate out and a few   smears were also examined to determine the stage of the malaria parasites at that particular hour

The protein fractions, the total proteins as well as the globulin   albumin ratio together with those physical properties that are likely to be influenced by these, such as the surface tension, the relative viscosity, the pH and the buffer action, were determined   For comparison a similar study was made on a number of cases where the blood was drawn after the rigor had subsided

Experimental procedure

All the determinations were made by the methods indicated in Part I of the present series (Chopra, Mukherjee and Rao, 1934)   The protein fractions were estimated at 17 5°C by the refractometric method as before   The amount precipitated by 33 per cent saturation with ammonium sulphate was regarded as the euglobulin   The data so obtained are tabulated below

RESULTS AND DISCUSSION

In order to compare the data as obtained for malarial cases similar figures for normal Indian subjects were determined and these have already been published in our previous paper   The normal averages obtained from this table are given in the last horizontal column in Table I

In Table I are also incorporated the results of examinations of blood of patients actually in a state of rigor   It will be observed that the pH and the buffer action of blood sera during the rigor remain very nearly the same as in normal individuals, while the surface tension and the relative viscosity are both lowered to a slight extent   As regards the proteins, however, the divergence from the normal are more marked, the total proteins and the albumin fractions being considerably lowered while the euglobulin fractions are raised to as much as 0 40 per cent in individual cases (average 0 30 per cent), as against 0 18 per cent in the average for normal persons   The pseudo-globulin fractions are not much affected, only a very few cases showing a slight decrease   The globulin   albumin ratio is fairly raised but it never exceeds unity   In the two cases marked with an asterisk the rigor was not very definite, only a chilly sensation being present when the blood was taken   In these two also the total proteins, the albumins as well as the globulin · albumin ratio were very nearly equal to those in the other rigor cases

In Table II have been incorporated the data from cases where the blood was drawn after the rigor had subsided   It is evident that the pH and the buffer action remained normal (*vide* Table II)   The surface tension was slightly raised and became very nearly equal to normal figures, the relative viscosity was still lowered although the amount of lowering was slightly smaller than in the previous cases

The total proteins were lower than those of normal persons but slightly higher than those obtained during the rigor state   The percentage of albumin, although much lower than that of normal persons, was slightly higher than in the rigor blood (Table I)   The euglobulin fraction remained, as during rigor, much higher than the normal figures   The pseudo-globulin fraction remained unchanged as before   In one patient marked with an asterisk, the blood was drawn when the temperature became normal after the attack of fever and in this case also the physical properties and the proteins were the same as in other cases (Table II)   This is in agreement with Lloyd and Paul's observations *(loc cit)* who found that the changes in the proteins in malarial cases persist for some time even when there is no fever

From a comparison of Tables I and II it would appear that although there is a difference in the average values of the corresponding items, these differences are so small that one would not be justified in drawing any conclusions   In order to verify these we studied blood sera from five patients both during and after rigor for the same individuals   The results have been given in Table III

Here also all our findings for the rigor blood are slightly different from those after the paroxysm and these differences corroborate our observations on the cases recorded in Tables I and II   Although the number of cases studied in this connection is relatively small, the findings all tend in the same direction and are definite and confirmatory   It is evident from the above that, as compared with normal figures, the total proteins in malarial sera, especially during and after the rigor state, are appreciably diminished   This diminution is due to a marked decrease in the percentage of albumin   The pseudo-globulin fractions change very little, while the euglobulins increase to an appreciable extent, in consequence of which the total globulins show an increase and the globulin   albumin ratio increases   The observations of Lloyd and Paul *(loc cit)* on the diminution of total proteins and albumin and the increase of euglobulin in the blood sera of chronic malarial patients differ from those of ours in as much as the changes as determined by these workers are more marked *(vide* Tables I, II and III)   These changes in the proteins, as our present work shows, are more prominent during the rigor stage than when the paroxysm is over

Of the physical properties studied herein the pH and the buffer action do not undergo any change as compared with the normal sera although there are considerable changes in the serum proteins   This may most probably be due to the comparatively less important part played by the proteins in maintaining the serum reaction

The relative viscosity is found to decrease only slightly as compared with the decrease of the total proteins   This is due to a slight increase in the euglobulin percentage which raises the viscosity of the serum much more than a corresponding amount of albumin or pseudo-globulin would do, since euglobulin is known to be associated with a much greater amount of water than either of the two other serum proteins (Chick, 1914)

Hence we have in these cases two opposing factors influencing viscosity, viz , the fall in albumin which decreases viscosity, and an increase in euglobulin which acts in the opposite direction   For this reason there is a small decrease in viscosity in these cases, although there is a comparatively larger decrease in the percentages of albumin or of the total proteins

Studies on the Protein Fractions of Blood Sera

TABLE I.

Blood drawn during the rigor state

Number	Parasites	pH	Buffer action 0.6 cc serum and 0.4 cc 0.01 N HCl	Buffer action 0.4 cc serum and 0.6 cc 0.01 N HCl	Relative viscosity	Surface tension in dynes	Total protein in g per cent †	Albumin in g per cent	Pseudo-globulin in g per cent †	Euglobulin in g per cent †	Globulin albumin ratio
1	Scanty quartan, growing trophozoites and gametocytes	7.41	6.99	6.82	1.55	57.2					
2	B.T. rings and growing trophozoites, scanty crescents	7.33	6.91	6.73	1.45	58.2					
3	M.T., B.T. and quartan rings, growing trophozoites, gametocytes of B.T. and Q.T.	7.47	7.14	6.85	1.55	58.2					
4	Scanty B.T. trophozoites	7.65		7.20	1.50	58.5					
5	B.T. rings and growing trophozoites, scanty gametocytes	7.43	7.10	6.80	1.44	58.2					
6	B.T. rings and crescents, M.T. and Q.T. gametocytes	7.40	7.05	6.81	1.40	58.9	6.46	3.50	2.65	0.31	0.846
7	B.T. growing trophozoites and gametocytes, scanty	7.47	7.10	6.80	1.31	59.6	6.73	3.90	2.61	0.22	0.726
8	M.T. rings and crescents	7.11	6.98	6.78	1.50	56.9	6.08	3.20	2.50	0.29	0.848

9	M T rings ++	7 66		6 96	1 48	59 1	5 98	3 10	2 63	0 25	0 929
10	Scanty quartan rings, growing trophozoites and gametocytes	7 47	7 21	6 91	1 48	58 6	6 11	3 31	2 40	0 40	0 846
*11	Scanty quartan gametocytes	7 50	7 15	6 93	1 51	59 3	6 80	3 95	2 57	0 28	0 722
12	B T growing trophozoites	7 49	7 22	6 86	1 55	59 5	6 10	3 57	2 20	0 33	0 737
*13	B T rings and growing trophozoites, schizonts and gametocytes	7 45	7 12	6 67	1 63	58 6	6 89	3 91	2 63	0 35	0 762
14	Q T rings, growing trophozoites, schizonts and scanty gametocytes	7 43	7 04	6 75	1 68	55 0	6 38	3 88	2 11	0 40	0 647
15	B T and Q T rings, growing trophozoites and schizonts	7 29	7 05	6 40	1 36	55 1	5 22	3 26	1 79	0 17	0 600
16	Quartan rings, growing trophozoites and schizonts	7 29	6 87	6 32	1 50	55 42	5 93	3 60	2 07	0 26	0 648
	Averages of rigor blood	7 15	7 07	6 73	1 49	57 9	6 24	3 57	2 38	0 30	0 756
	Averages of normal Indian blood	7 48	7 14	6 88	1 60	60 1	7 44	4 61	2 63	0 18	0 611

* In this case the blood was drawn after the fever had subsided and the temperature had become normal

† g per cent indicates the number of grammes per 100 c c of serum

TABLE II

Blood drawn after the rigor had subsided

Number	Parasites	pH	Buffer action 0 6 c c serum and 0 4 c c 0 01 N HCl	Buffer action 0 4 c c serum and 0 6 c c 0 01 N HCl	Relative viscosity	Surface tension in dynes	Total proteins in g per cent †	Albumin in g per cent †	Pseudo-globulin in g per cent †	Euglobulin in g per cent †	Globulin albumin ratio
1	Scanty M T rings and crescents	7 50	7 20	6 96	1 61	56 7	6 18	3 20	2 69	0 29	0 931
2	Scanty M T rings and crescents	7 40	7 10	6 90	1 66	56 6	6 54	3 52	2 96	0 36	0 943
3	No parasites in the periphery	7 39	7 10	6 90	1 50	58 3	6 32	3 59	2 41	0 32	0 760
4	Scanty M T rings	7 47	7 13	6 80	1 63	59 3	6 18	3 31	2 53	0 33	0 864
5	Scanty M T rings	7 45	7 20	6 95	1 55	58 5	6 20	3 66	2 50	0 31	0 762
6*	B T growing tropho zoites and very scanty gametocytes	7 57	7 18	6 73	1 54	61 1	6 99	4 22	2 44	0 33	0 684

	Description									
7	B T and scanty M T rings and growing tro phorotes	7 37	6 95	6 74	1 36	61 2	6 86	4 40	2 73	0 16	0 722
8	Scanty B T rings and Q T rings and scanty gametocytes	7 60	7 29	6 97	1 40	61 0	6 63	3 74	2 69	0 20	0 773
9	Q T rings, growing trophozoites, schizonts and scanty gameto cytes	7 56	7 00	6 80	1 50	56 3	6 71	4 25	2 14	0 32	0 579
10	Q T rings, growing trophozoites and schizonts	7 43	7 10	6 81	1 55	56 7	5 52	3 50	1 85	0 17	0 577
	Averages	7 47	7 12	6 86	1 53	58 6	6 50	3 70	2 49	0 28	0 760
	Averages of rigor blood	7 45	7 07	6 73	1 49	57 9	6 24	3 57	2 38	0 30	0 756
	Averages of normal Indian blood	7 48	7 14	6 88	1 60	60 1	7 44	4 61	2 63	0 18	0 611

* In this case the blood was drawn after the fever had subsided and the temperature had become normal

† g per cent indicates the number of grammes per 100 c c of serum

TABLE III

Blood drawn during and after rigor for the same individuals

Number	Parasites	pH	Buffer action 0.6 cc serum and 0.4 c.c serum 0.01 N HCl	Buffer action 0.1 c.c serum and 0.6 c.c 0.01 N HCl	Relative viscosity	Surface tension in dynes	Total protein in g per cent*	Albumin in g per cent*	Pseudo-globulin in g per cent*	Euglobulin in g per cent*	Globulin albumin ratio
1	B T rings and trophozotes—										
	(a) during rigor	7 33	6 91	6 73	1 15	58 2					
	(b) after rigor	7 40	7 10	6 98	1 45	58 5					
2	B T, M T and Q T rings, growing trophozoites and gametocytes of B T and Q T—										
	(a) during rigor	7 47	7 14	6 85	1 45	58 2					
	(b) after rigor	7 61	7 25	7 05	1 55	59 5					
3	Q T rings, growing trophozoites, schizonts and scanty gametocytes—										
	(a) during rigor	7 43	7 05	6 75	1 68	65 0	6 39	3 88	2 11	0 40	0 647
	(b) after rigor	7 56	7 10	6 80	1 50	56 3	6 71	4 25	2 14	0 32	0 579
4	B T and Q T rings and growing trophozoites—										
	(a) during rigor	7 36	7 00	6 40	1 36	55 1	5 22	3 26	1 79	0 17	0 600
	(b) after rigor	7 56		6 80	1 50	56 7	5 52	3 50	1 85	0 17	0 577
5	Q T rings, growing trophozoites and schizonts—										
	(a) during rigor	7 29	6 87	6 32	1 50	55 4	5 93	3 60	2 07	0 26	0 648
	(b) after rigor	7 40	7 05	6 72	1 60	57 2	6 24	3 89	2 17	0 18	0 604

* g per cent indicates the number of grammes per 100 c c of serum

As regards the surface tension both during and after the rigor state the figures were slightly lower than the normal figures From the decrease in the protein content one would expect a rise in the surface tension and therefore such values of surface tension as were found in these cases may be due to some other factor or factors It is possible that the increase in the percentages of euglobulin as observed in these cases may be responsible for this state of affairs, inasmuch as euglobulin is known to be associated with a large amount of water which raises the effective concentration of the proteins in the whole serum

It is also evident in these cases that the changes in the physical properties and the protein fractions are always more prominent during the rigor state than after it From our observations it appears to be likely that the changes in the blood sera of malarial patients that persist even after the rigor state is over, really take place during the rigor state They gradually begin to disappear as soon as the paroxysm is over, probably returning to their normal values in a few days after the fever subsides The above contention appears to us to be probable in view of the fact that the rigor is a severe shock probably of protein nature which affects the heat centres leading to a sudden and high rise of temperature Whether these proteins come from sporozoites which are liberated or whether they are produced by the system in response to the infection is difficult to say

SUMMARY AND CONCLUSION

During the rigor state in malaria the physical properties such as the pH and the buffer action change very little, while the relative viscosity and the surface tension are both lowered, the former to a greater extent than the latter

The protein fractions all deviate from the normal, albumin diminishes considerably, the euglobulin increases to a certain extent while the pseudo-globulin remains practically normal The total proteins also diminish to a considerable extent In those cases where the blood was drawn after the rigor had subsided the changes in the physical properties and also in the proteins are similar to those of the rigor cases but such changes are less marked and more towards normal

From these we are led to conclude that the changes in the physical properties as well as in the proteins of blood sera in malarial patients really set in during the rigor and reach a maximum when these changes begin to disappear and finally reach normal values within a short period after the rigor is over

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THE EFFECT OF EXPOSURE OF SUSPENSIONS OF RABIES-INFECTED BRAIN TO RADIATION FROM A QUARTZ MERCURY VAPOUR LAMP

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THE preparation of rabies vaccine is still an unsatisfactory procedure. Apart from the undesirability of introducing foreign brain tissue into the body, the method of inactivating the virus by carbolic acid leaves much to be desired   Hence we find that attempts have been made to inactivate the brain suspension by means of ether and formalin   A new avenue of research was opened up by Clifton's (1931) work on the inactivation of certain bacteriophages by the photodynamic action of methylene blue   Perdrau and Todd (1933a and 1933b) confirmed and extended this work by recording similar inactivation in the case of other animal viruses   vaccinia, herpes, fowl plague, louping ill, borna disease, and canine distemper   The most important result of these researches was that noted by Perdrau and Todd (1933c), who showed that the virus of distemper, thus inactivated, preserved its antigenic property and could be used as an efficient vaccine   The practical application of this phenomenon is of far reaching importance in the therapy of virus diseases

Shortt and Brooks (1934) investigated the photodynamic action of methylene blue on the fixed virus of rabies , they found that the virus was inactivated thereby   Almost simultaneously Galloway (1934) confirmed Shortt and Brooks' work , in addition he proved that the inactivated virus retains its antigenic potency

From the results reported by the observers, it is seen that the concentration of infective material used by them was very low   Thus, Shortt and Brooks used a 0 5 per cent suspension after its centrifugalization for 2 minutes in a hand centri-fuge , a procedure which resulted in the titre of the virus being far below 0 5 per cent   Galloway was unable to inactivate 0 5 to 1 25 per cent suspensions that were centrifuged for 10 minutes at 2,500 revolutions per minute , but he could only do so when they were filtered through ' sand and paper filter '   Such filtrates were fairly clear ' and had an infectivity of approximately one-tenth that of a centrifuged

unfiltered suspension' In other words, Galloway succeeded in inactivating a rabies infected brain suspension only when the concentration was as low as 0 05 to 0 125 per cent In view of the fact that Pasteur Institutes in India find it necessary to use as high a concentration of virus as a 5 per cent uncentrifuged brain suspension, and that the results of the researches on the photodynamic inactivation of the virus have shown that such inactivation does not occur in suspensions of a concentration higher than 0 5 per cent, it seemed that the results had no immediate practical application The object of the investigation reported in this paper was to devise means for the inactivation of the 5 per cent brain suspension now in general use in India

The explanation given by Perdrau and Todd (1933a) for their failure to inactivate unfiltered suspensions by the above means was that protection was afforded to the virus by living cells But the fact that Shortt and Brooks could inactivate an opalescent, centrifuged suspension which, 'under a microscopic examination with high powers, showed an abundance of cellular material ', seems to indicate that no protection is afforded to the virus by living cells Another explanation put forward by Perdrau and Todd was that the screening effect of even relatively thin layers of the methylene blue solution may have protected the virus from the photodynamic action of light Shortt and Brooks accept the latter explanation, for they found that while a higher concentration of the dye-stuff, such as 1 in 10,000 prevented inactivation, a lower concentration (1 in 200,000) favoured it

Theoretical considerations led one of us (G S) to think that for light to penetrate through a blue solution a shorter wave-length might be needed Further, Shortt and Brooks made use of daylight in their experiments, while the other workers employed incandescent filament and pointolite lamps It seemed likely that the success of Shortt and Brooks in inactivating unfiltered suspensions was perhaps due to the presence in the daylight of rays of short wave-lengths, such as the ultra-violet, which are absent in light from filament and pointolite lamps

Our first experiment was designed to test this theory spectrographically The rays from a quartz mercury vapour lamp were passed through a quartz cell of one centimetre length containing various concentrations of methylene blue and unfiltered brain suspensions, in order to see whether a photographic plate was affected by the light traversing these mixtures It was found that a mixture of a 1 per cent centrifuged brain suspension with a 1 in 50 000 concentration of methylene blue was freely pervious to ultra-violet light of wave-length from 3,000 to 1,800 A U Encouraged by this result we decided to repeat the experiments of the previous workers, but using a mercury vapour lamp as the source of light for the photodynamic action

METHODS

The material —For each experiment fresh suspensions were made of rabies infected brain material from rabbits In some experiments infected brain preserved in 50 per cent glycerine was used The strain of the virus was the fixed virus, Paris, kept in successive sub-culture through rabbits at this Institute for the last two years A few experiments were performed with street virus received in this Institute from various parts of Southern India The street virus brain material was injected intramuscularly into guinea-pigs, and when the animals had developed rabies their brains were used for our experiments In our early experiments we

used a 0 5 per cent eoneentration of infective material in distilled water, centrifuged in an electric eentrifuge for 10 minutes   In later experiments we stopped centrifuging the suspension and progressively increased the eoneentration to 5 per eent   In these later experiments we used physiological saline for making the suspensions   In this paper a suspension of rabies infected brain is referred to as ' virus suspension ', inasmuch as the term ' emulsion ' is, from its meaning, inapplicable to a suspension of brain tissue

The dye —The dye used was methylene blue   It was supplied by Messrs Baird and Tatloek and was marked ' Methylene Blue (Koch) '   It had been in the store for some years in an unopened bottle   Of this we used a 1 in 25,000 solution which on admixture with equal parts of virus suspension gave a eoneentration of the dye of 1 in 50,000   The methylene blue solution was autoelaved before each experiment   In the latter experiment the dye was made up in physiological saline

The lamp —The souree of the ultra-violet light was a quartz mercury vapour lamp (Heræus) working on a voltage of 220 direct eurrent   It is capable of giving a 2,000 candle power light working on full load of 10 amperes , but by a regulator starter it was worked for safety at a load of 5 amperes, giving an intensity of light of approximately 1,000 candle power

The technique of irradiation —Five cubic centimetres of virus suspension was introduced into a sterile Petri dish, of 4-inch diameter, by means of a sterile pipette   To this were added five cubic eentimetres of the sterile methylene blue solution made up eithei in distilled water or in saline   By this means a eonstant depth of the suspension was assured   No particular care was taken to do the mixing in a dark-room   The Petri dish· was, however, always protected from daylight by placing it in a covered enamelled bowl   The material to be irradiated was placed 10 inehes from the mereury vapour lamp   The time of exposure was noted with a stop-eloek   There was not mueh heating effeet due to the light   This was demonstrated by placing a thermometer just above the suspension   It was found to register a temperature of 10°C to 14°C higher than the outside air, after an exposure of 30 minutes to the rays from the mercury vapour lamp   The actual temperature of 10 cubie eentimetres of the suspension was found to be raised by only 4°C   Since the temperatures at which the work was done (24°C to 34°C) were far below body temperatures, no special preeautions were taken to absorb the heat rays , moreover, such attempts would have cut off all ultra-violet light unless costly optieal quartz absorbing vessels had been used

Animal inoculation —-The animal used in all our experiments was the rabbit   The subdural method of inoeulation was employed   this being more reliable than the intramuseular method   This proeedure is a severe test   The amount of inoculum was 0 2 c c   The symptoms of rabies develop, as a rule, in from 7 to 9 days following the subdural inoculation , in rare cases, however, they may not appear until 14 to 21 days   Our animals were, accordingly, kept under observation for two months

EXPERIMENTS AND RESULTS

Full details of the experiments and their results are given in the aecompanying Tables —

Effect of Radiation on Rabies-infected Brain.

TABLE I

Showing results of experiment I

Date	Concentration of virus suspension.	Concentration of methylene blue	Duration of irradiation (in minutes)	Serial number of rabbits inoculated	Died of rabies	Alive and well after 2 months	Remarks
25-7-34	0 5 per cent in distilled water Fixed virus			1	6th day		Control
	do	1 in 50,000	10	2, 3	6th ,,		Suspension mixed in the dark
	do		20	4, 5		4, 5	
	do	1 in 50,000	15	6, 7		6, 7	
	do	1 in 50,000	30	8, 9		8, 9	
	do	1 in 50,000	15	10, 11		10, 11	
	do		30	12, 13	6th day		
	do	1 in 50,000	15	14, 15	6th		The methylene blue was irradiated and then added to the virus suspension in the dark
	do	1 in 50,000	30	16, 17		16, 17	do
	do			18, 19		18	The virus was irradiated and then added to the methylene blue solution
							No 19 died of disease other than rabies on the 38th day

TABLE II

Showing results of experiment II

Date	Concentration of virus suspension	Concentration of methylene blue	Duration of irradiation (in minutes)	Serial number of rabbits inoculated	Died of rabies	Alive and well after 2 months	Remarks
12-0-34	0 5 per cent in distilled water Fixed virus			56 57	} 6th day		Control
	do	1 in 50,000	2	58 59 60		58 59 60	
	do		2	61 62 63		61 62 63	
	do	1 in 50,000	5	64 65 66		64 65 66	
	do		5	67 68 69		67 68 69	
	do	1 in 50,000	10	70 71 72		70 71 72	
	do	1 in 50,000	10	73 74 75		73 74 75	
	do		15	76 77 78		76 77 78	
	do	1 in 50,000	15	79 80 81		79 80 81	

TABLE III.

Showing results of experiment III

Date.	Concentration of virus suspension	Concentration of methylene blue	Duration of irradiation (in seconds)	Serial number of rabbits inoculated	Died of rabies	Alive and well after 2 months	REMARKS
	0 5 per cent in distilled water Fixed virus	.		136 137	}7th day		Control
	do	1 in 50,000	15	112 113 114	.	112 113 114	
	do	.	15	115 116 117	11th day	115 117	
	do	1 in 50,000	30	118 119 120	12th day	118 120	
21-6-34	do	..	30	121 122 123	.	121 122 123	
	do		60	124 125 126		124 125 126	
	do	in 50,000	60	127 128 129		127 128 129	
	do		90	130 131 132	.	130 131 132	
	do	1 in 50,000	90	133 134 135	. .	133 134 135	

TABLE IV

Showing results of experment IV

Date	Concentration of virus suspension	Concentration of methylene blue	Duration of irradiation (in minutes)	Serial number of rabbits inoculated	Died of rabies	Alive and well after 2 months	Remarks
31-5-34	0 5 per cent in distilled water Street virus			20	11th day		Control
	do			21	11th day		
	do		20	22		22	
	do		20	23		23	
	do		40	24		24	
	do		40	25		25	
	do	1 in 50,000	20	26		26	
	do	1 in 50,000	20	27		27	
	do	1 in 50,000	40	28		28	
	do	1 in 50,000	40	29		29	

TABLE

Showing results of experiment V *

Date	Concentration of virus suspension	Concentration of methylene blue	Duration of irradiation (in minutes)	Serial number of rabbits inoculated	Died of rabies	Alive and well after 2 months	Remarks
	0 5 per cent in distilled water Street virus			82 83	12th day 10th ,,		Control
	do		2	84 85 86		84 85 86	Died of disease other than rabies—33rd day
	do	1 in 50,000	2	87 88 89		88 89	Died of disease other than rabies—26th day
	do		5	90 91 92		91 92	Died of disease other than rabies—58th day
13-6-34	do	1 in 50,000	5	93 94 95		93 95	Died of disease other than rabies—39th day
	do		10	96 97 98 99		96 98 99	Died of disease other than rabies—33rd day
	do	1 in 50,000	10	100 101 102		100	Died of disease other than rabies—33rd day
	do		15	103 104 105		104 105	Died of disease other than rabies—53rd day
	do	1 in 50,000	15	106 107 108		106 107 108	

* In this experiment we seem to have used an unhealthy group of rabbits The animals that died while under observation were infected with coccidiosis and a condition of mango Brain and animal inoculation proved negative for rabies

TABLE VI

Showing results of experiment VI

Date	Concentration of virus suspension	Concentration of methylene blue	Duration of irradiation (in minutes)	Serial number of rabbits inoculated	Died of rabies	Alive and well after 2 months	Remarks
14-6-34	5 per cent fixed virus in saline		15	108a	7th day		
	do			109		109	Control
				110	9th day ,,		
				111	11th ,,		

TABLE VII

Showing results of experiment VII

During irradiation the Petri dish containing the suspension was rocked by hand

Date	Concentration of virus suspension	Concentration of methylene blue	Duration of irradiation (in minutes)	Serial number of rabbits inoculated	Died of rabies	Alive and well after 2 months	Remarks
6-7-34	5 per cent fixed virus in distilled water			142	7th day		
	do		20	143	7th ,,		
	do	1 in 50,000	20	138		138	Control
				139		139	
				140		140	
				141		141	

TABLE VIII

Showing results of experiment VIII

During irradiation the Petri dish containing the suspension was rocked by an electric motor

Date	Concentration of virus suspension	Concentration of methylene blue	Duration of irradiation (in minutes)	Serial number of rabbits inoculated	Died of rabies	Alive and well after 2 months	REMARKS
6-8-31	5 per cent fixed virus in brine			166 167	7th day		Control
	do		20	154 155 156		154 155 156	
	do		10	157 158 159		157 158 159	
	do		5	160 161 162		160 161	
	do		2	163 164 165	8th day	163	Died of disease other than rabies—45th day
						165	

In the *first experiment*, the following materials were used for subdural inoculation —

1   A mixture of equal parts of a 1 per cent suspension of fixed virus in distilled water and of a 1 in 25,000 solution of methylene blue The mixture was made in the dark and allowed to stand for 20 minutes before it was inoculated

2   A mixture of equal parts of a 1 per cent suspension of fixed virus and distilled water irradiated for 10 minutes

3   A mixture of equal parts of a 1 per cent suspension of fixed virus and distilled water irradiated for 20 minutes

4   A mixture of equal parts of a 1 per cent suspension of fixed virus and a 1 in 25,000 solution of methylene blue irradiated for 15 minutes

5   A mixture of equal parts of a 1 per cent suspension of fixed virus and a 1 in 25,000 solution of methylene blue irradiated for 30 minutes

6   A 1 in 25,000 solution of methylene blue irradiated for 15 minutes, to which was added, in the dark, an equal amount of an unirradiated 1 per cent suspension of fixed virus in distilled water

7   A 1 in 25,000 solution of methylene blue irradiated for 30 minutes, to which was added, in the dark, an equal amount of an unirradiated 1 per cent suspension of fixed virus in distilled water

8   A suspension of fixed virus in distilled water irradiated for 15 minutes, to which was added an equal amount of unirradiated 1 in 25,000 solution of methylene blue

9   A suspension of fixed virus in distilled water irradiated for 30 minutes, to which was added an equal amount of unirradiated 1 in 25,000 solution of methylene blue

Two animals were inoculated with each of these materials Those inoculated with 1, 6 and 7 died of rabies, those inoculated with 2, 3, 4, 5, 8 and 9 did not develop rabies A control animal inoculated with unirradiated virus suspension developed rabies on the sixth day

In the *second experiment*, the following materials were used for subdural inoculation —

(1)   A suspension of fixed virus in distilled water, irradiated for 2 minutes

(2)   Equal parts of a fixed virus suspension and a 1 in 25,000 solution of methylene blue, irradiated for 2 minutes

(3)   Same as (1) but irradiated for   5 minutes

(4)   do   (2)   do   5   do

(5)   do   (1)   do   10   do

(6)   do   (2)   do   10   do

(7)   do   (1)   do   15   do

(8)   do   (2)   do   15   do

Three animals were inoculated with each of these materials None developed rabies As a control, two animals were inoculated with the unirradiated virus suspension, both developed rabies on the sixth day

The *third experiment* was similar to the second except that irradiation was for periods of 15, 30, 60 and 90 seconds   The result of the experiment was as follows   Complete inactivation of the virus suspension resulted from 60 and 90 seconds irradiation   Of the 6 animals inoculated with material irradiated for 15 seconds only one developed rabies   and that belonged to the group inoculated with a mixture of virus suspension and irradiated solution of methylene blue   Of the 6 animals inoculated with virus suspension that had been irradiated for 30 seconds, only one developed rabies and that belonged to the group inoculated with irradiated virus suspension to which no methylene blue had been added

The *fourth and fifth experiments* were similar to the second and third, except that street virus was used instead of fixed virus   Irradiation was for 2, 5, 10, 15, 20 and 40 minutes   None of the animals inoculated with virus suspensions so irradiated developed rabies , while all animals inoculated with the same virus suspension that had not been irradiated did develop rabies

We were thus led to the conclusion that we were dealing with the action of ultra-violet light alone   The methylene blue appeared to have no effect   Encouraged by these results, obtained with irradiated 0 5 per cent virus suspension, we next attempted to inactivate a 5 per cent suspension of the fixed virus

In the *sixth experiment* a 5 per cent suspension of the fixed virus was prepared in sterile normal saline, and filtered through two layers of sterile gauze , it was then irradiated for 15 minutes   Three animals were inoculated with 0 2 c c of the irradiated suspension   Two animals developed rabies   one on the ninth and the other on the eleventh day   The third animal did not develop rabies   The delay in the occurrence of rabies, although slight, suggested the possibility of some attenuation of the virus   The failure in this experiment to inactivate the virus was probably due to the fact that the ultra-violet rays were not able to penetrate the whole of the 5 per cent suspension   At the suggestion of Sir Robert McCarrison we adopted the method of rocking the suspension in the Petri dish to and fro while irradiation was taking place   We also repeated our experiments with methylene blue

In the *seventh experiment* a five per cent suspension of fixed virus, in sterile distilled water, filtered through two layers of sterile gauze, was irradiated for 20 minutes , the suspension in the Petri dish being rocked to and fro by hand during the period   Complete inactivation occurred with and without the methylene blue

We devised a mechanical shaker, worked by an electric motor, thus avoiding the bad effects of the exposure of the hand to ultra-violet light   The diagram and photograph (Plate XXII, figs 1 and 2) explain the principle of this shaker   An eccentric wheel raises one end of the platform on which the Petri dish is placed   The motor that rotates the eccentric wheel is a universal electric one whose speed can be regulated as required   A comfortable rate of rocking was about 10 to 15 oscillations per minute

The *eighth experiment* was carried out with the aid of this rocker   A 5 per cent virus suspension in saline was irradiated for 2, 5, 10 and 20 minutes   Twelve animals were inoculated with the irradiated material, one of these developed rabies   It was one of the three that were inoculated with virus suspension irradiated for 5 minutes   The other 11 animals are alive and well   Two control animals inoculated with the same virus suspension that had not been irradiated died of rabies on the seventhday following inoculation

PLATE XXII
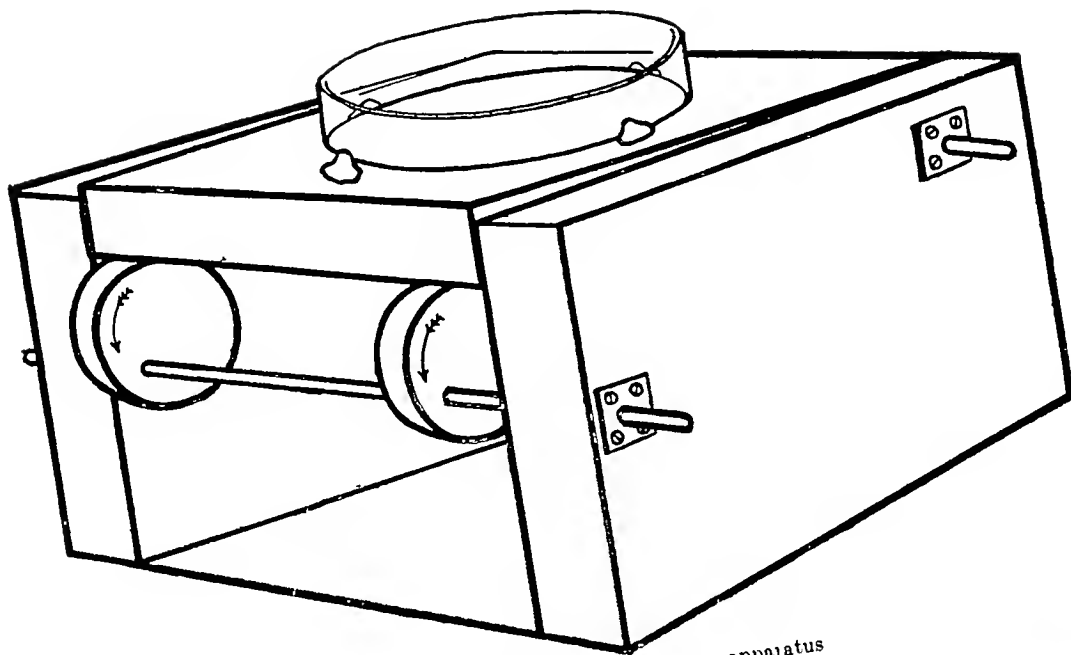
Fig 1—Diagram of the rocking apparatus
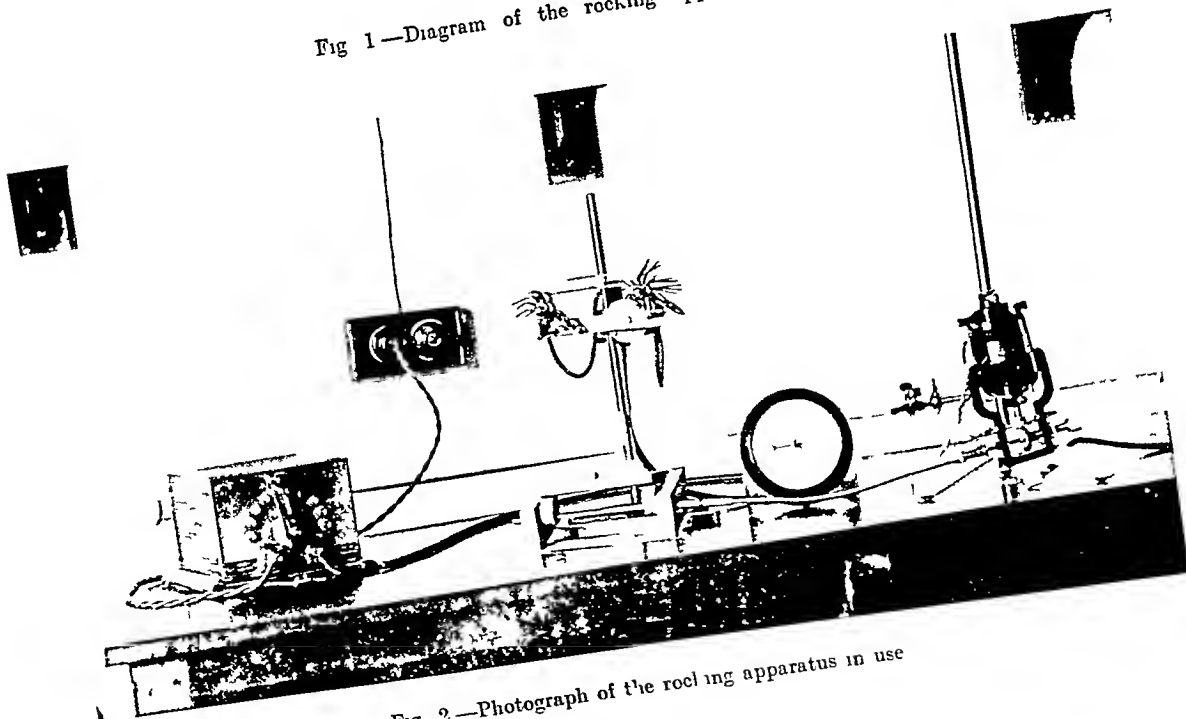
Fig 2—Photograph of the rocking apparatus in use

For irradiation of large amounts of vaccine other methods, such as passing the vaccine in a thin film over glass plates in a slow stream or a quartz rod or tube with a mercury arc inside might be used as a stirrer in a large bulk of the suspension   Both these methods will be tried shortly

DISCUSSION

These results demonstrate that exposure of a 5 per cent suspension of rabies infected brain to ultra-violet light, or more precisely to light from a mercury vapour lamp, inactivates the rabies virus in 10 minutes, provided proper precautions as to oscillation of the suspension are taken   These results appear to us to be of greater practical significance than the photodynamic action of methylene blue   The inactivation is easily carried out and the addition of extraneous chemical substances is unnecessary   Whether immunizing substances are preserved under the above circumstances is a problem that is now engaging our attention, and if we find that they are preserved, there is no doubt that we have a method of making rabies vaccine which is far quicker and simpler than the methods now adopted   At present it is necessary to wait for nearly 6 to 7 weeks before a carbolized vaccine can be issued for use   It is accepted that the antigenic property of a vaccine deteriorates with age   A vaccine made out of infected rabies brain which has been irradiated for 10 minutes is immediately available for use, and it may be assumed that because of its freshness it ought to have greater antigenic property than one 7 weeks old   Experiments are in progress to test this view

As to the causes of inactivation there are at present several theories   A mercury vapour lamp gives radiation of wave-length from 6,500 A U to very nearly 1,800 A U   We hope to investigate at what particular wave-length the inactivation is greatest   There is some evidence that the shorter wave-lengths of light, such as ultra-violet 4,000 to 1,800, are highly inactivating   It is probably the large preponderance of these wave-lengths in the mercury vapour lamp that accounts for the inactivation and not the relatively large intensity of visible light from 4,000 to 6,500 A U that is also present to the extent of nearly 1,000 candle power   Further, Downes and Blunt, as early as 1877, believed that oxygen was essential for germicidal action of light   Arloing (1885) thought that it was due to its effect on the oxidizing reactions   Duclaux (1885) and Roux (1887) concur in this conclusion   Methylene blue was said to form a peroxidase under the influence of ultra-violet light which was suspected to cause the inactivation of the virus   To test this view we irradiated methylene blue in our first experiment and then added it to the virus   Our results disproved the theory

Another reason for the inactivation caused by ultra-violet light might be the ozone generated by the mercury vapour lamp   Experiments are in progress to see if ozone bubbled through a virus suspension will cause its inactivation

The influence of H-ion concentration on the inactivating property of light has been noted by Bayne-Jones and van der Lingen (1923)   An acid medium is more favourable for the destructive action than an alkaline one   We are now investigating this problem

Some recent speculations regarding the action of ultra-violet light from a physico-chemical view-point are likely to throw more light on the mechanism of inactivation of viruses   It is well known that living protoplasm and many proteins

absorb ultra-violet light   Lipoids especially are very powerful absorbers of it What seems to happen is that both inorganic and organic colloids undergo changes as a result of exposure to ultra-violet light   These changes are accompanied by a change in the electrical charges on the particles   Our recent evidence (Sankaran *et al* , 1934) that rabies virus possesses an electrical charge fits in with this theory

Clark (1923) has developed a photo-electrical theory postulating that electrons are given off from proteins under the action of ultra-violet light and that these electrons attach themselves to other atoms or molecules with resulting changes in both physical and chemical properties of substances concerned   We can with equal justification assume that such changes may bring about changes in physiological and pathological properties

CONCLUSION

(1) A 5 per cent suspension of rabies infected brain was successfully inactivated by irradiation for 10 minutes with the rays from a mercury vapour lamp the suspension being oscillated while the irradiation was in progress

(2) Methylene blue was unnecessary for inactivation

(3) The cause of inactivation is under investigation

(4) Experiments are in progress to see if the immunizing bodies are preserved after irradiation

We desire to express our indebtedness to Major K R K Iyengar, I M S, Director, Pasteur Institute, Coonoor, for affording facilities for work at the Institute and to Major-General Sir Robert McCarrison for his constant interest and advice

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FURTHER OBSERVATIONS ON INDIAN RELAPSING FEVER

Part II

THE SEROLOGY OF RELAPSING FEVER IN HUMAN BEINGS

CORRIGENDUM

In Volume **22,** No 3, January 1935 issue of the *Indian Journal of Medical Research*, page 539, under *Titration of serum* in line 2 of the para after ' tests ' *add* ' following the technique by S D S Greval (*Ind Jour Med Res*, **20,** 3, p 913)

dealt

... ... types of spiroc .... occurring in experimental infections in animals   Our object in the present paper is to see to what extent the conclusions reached by this means are applicable to the disease as it occurs in human beings

Our original intention was to investigate this question in two ways   (1) the examination of spirochætes derived directly from human cases of the disease to see if the types thus isolated corresponded with those already discovered in attacks and relapses in animals, and (2) the search for specific agglutinins to the various types of spirochæte in serum taken from human cases at different periods of the disease   Unfortunately, our intention with regard to the first of these two methods was to a great extent frustrated by the almost total absence of the disease† during the four years our investigation was in progress in Northern India   Occasional sporadic cases occurred in different parts of the country but we found it extremely difficult to reach them in time to obtain successful sub-inoculations, and we only succeeded in doing so on three occasions throughout the whole of this period

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*Frequent references will be made to this paper in the present communication , the actual page to which reference is being made will be given in brackets

† No epidemic occurred in either the United Provinces or the Punjab while we were working in North India   It is of interest to note, too, that since the 1923 24 epidemic in South India Dr Theodore reports that he has been unable to trace any cases of the disease in the Madras Presidency although he has been anxious to obtain another strain of spirochæte and the District Health Officers have been on the look out for such cases

Reference has already been made to the spirochætes isolated from these three cases (strain ' Punjab 1 ', strain ' Punjab 2 ', and strain ' Hazro ') (Cunningham *et al*, 1934, p 120).   Cross-agglutination tests carried out with the sera derived from experimental infections in monkeys with each of the three strains and the different types of spirochæte already obtained from the Madras strain proved the identity of strain ' Punjab 1 ' with Madras type ' A ', strain ' Punjab 2 ' with Madras type ' B ', and strain ' Hazro ' with Madras type ' C ' (Charts 1 and 2) No final conclusions can be drawn from these three instances alone, but it is, at any rate, significant that all three strains conformed serologically to one or other of the types of the Madras strain already in our possession

(2) As the first part of the investigation proved inconclusive we had to rely almost entirely upon the indirect evidence supplied by the examination of sera taken from patients at different stages of the disease

The material upon which this part of the investigation has been based was derived from three sources   (a) A severe epidemic of louse-borne relapsing fever which swept through the Nilgiri Hills in the year 1924   (b) Sporadic cases already mentioned as occurring in different parts of the Punjab between the years 1925 and 1929   (c) Cases of the disease occurring in different areas on the North-West Frontier

(a) *South Indian series* —A short reference to these was made in our original communication (Cunningham, 1925)   At the time this research was carried out only one type of spirochæte had been recognized [the original type isolated, viz , type ' A ' (at that time called ' first attack ' spirochæte)]   The majority of the tests were carried out with this type alone   Towards the end of this part of the investigation, however, the so-called ' relapse ' spirochæte (type ' B ') was recognized as a separate entity and a few sera were tested against both types   Since that time many other types have been isolated and our views have been considerably modified It has, therefore, been considered of value to reconsider these earlier results in the light of our present knowledge

The results were presented in the form of a chart which has been reproduced (Chart 3)   The agglutinin content of the serum of each patient (to type ' A ' spirochæte) has been charted against the particular stage of the disease at which the sample of blood was taken   The few instances in which the sera were also tested against the ' relapse ' spirochæte (type ' B ') have been especially marked   By this means it was possible to see how far the agglutinin content of the sera as a whole conformed to the curves obtained in experimental animals (in this case a composite curve derived from several monkeys to spirochætes ' A ' and ' B ')

The various observations have been charted with reference to the stage of the disease and not according to the time which has elapsed since infection   This was done to diminish the error arising from the varying lengths of the different stages of the disease in different individuals   For the same reason the observations on the monkey's curve cannot be regarded as absolutely fixed points when viewed in conjunction with the human sera   Thus, any day from the fourth to the tenth may mark the end of the first attack in the human series, so that the last day (fourth) in the case of the monkey's may be said to correspond to any position between these two days

CHART 1

Showing the agglutinin content of the serum of a monkey infected with strain 'Punjab 1' to the various types of spirochæte obtained from the Madras strain

DAY OF INFECTION   1 2 3 4 5 6 7 8 9 10   15   20   25   30   35   40   45   50   55

SPIROCHAETES IN BLOOD

SERUM TITRE

40,960
20,480
10,240
5,120
2,560
1,280
640
320
160
80
40
20
10
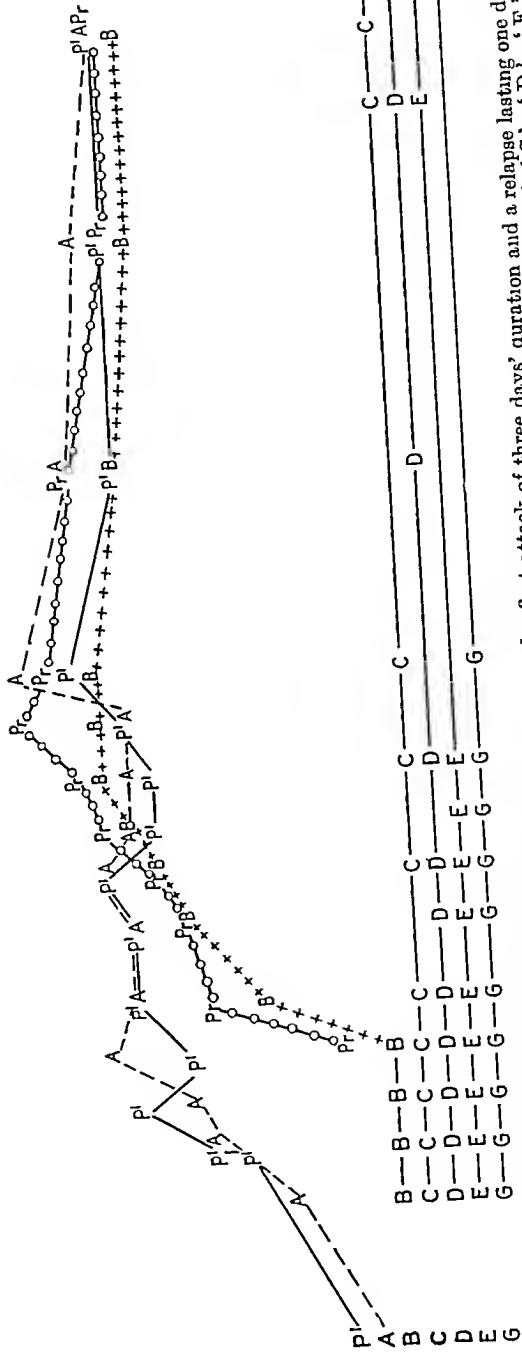
Monkey No 182 was infected with human strain 'Punjab 1' and showed a first attack of three days' duration and a relapse lasting one day five days later. Samples of serum taken at various periods up to the 590th day were tested against Madras strain types ('A', 'B', 'C', 'D', 'E', 'G'), and the 'Punjab 1' strain. The agglutinin curves are given in the chart and show that the 'Punjab 1' strain is identical with the Madras strain for 420 days. The spirochæte appearing in the relapse is obviously identical with Madras type 'B'. Type 'B' anti bodies remained in the serum for those for type 'A' (and 'Punjab 1') were present for 590 days

P¹ = 'Punjab 1' strain, Pr = the curve to the 'Punjab 1' strain 'relapse' spirochæte

CHART 2

Showing the agglutinin content of the serum of a monkey infected with strain 'Punjab 2' to the various types of spirochæte obtained from the Madras strain

DAY OF
INFECTION   1 2 3 4 5 6 7 8 9 10   15   20   25   30   35   40   45   50

SPIROCHAETES
IN BLOOD

SERUM TITRE

40,960
20,480
10,240
5,120
2,560
1,280
640
320
160
80
40
20
10

P_2 P_2 P_2 P_2 P_2 P_2 P_2B P_2B P_2B P_2
B   B   B   B   B B   B   B

P_2
A
B
C
D
E
G

A—A—A   A—A   A   A   A   A
C—C   C   C   C   C   C
D—D—D   D   D   D   D
E—E   E   E   E   E
G   G   G   G   G   G
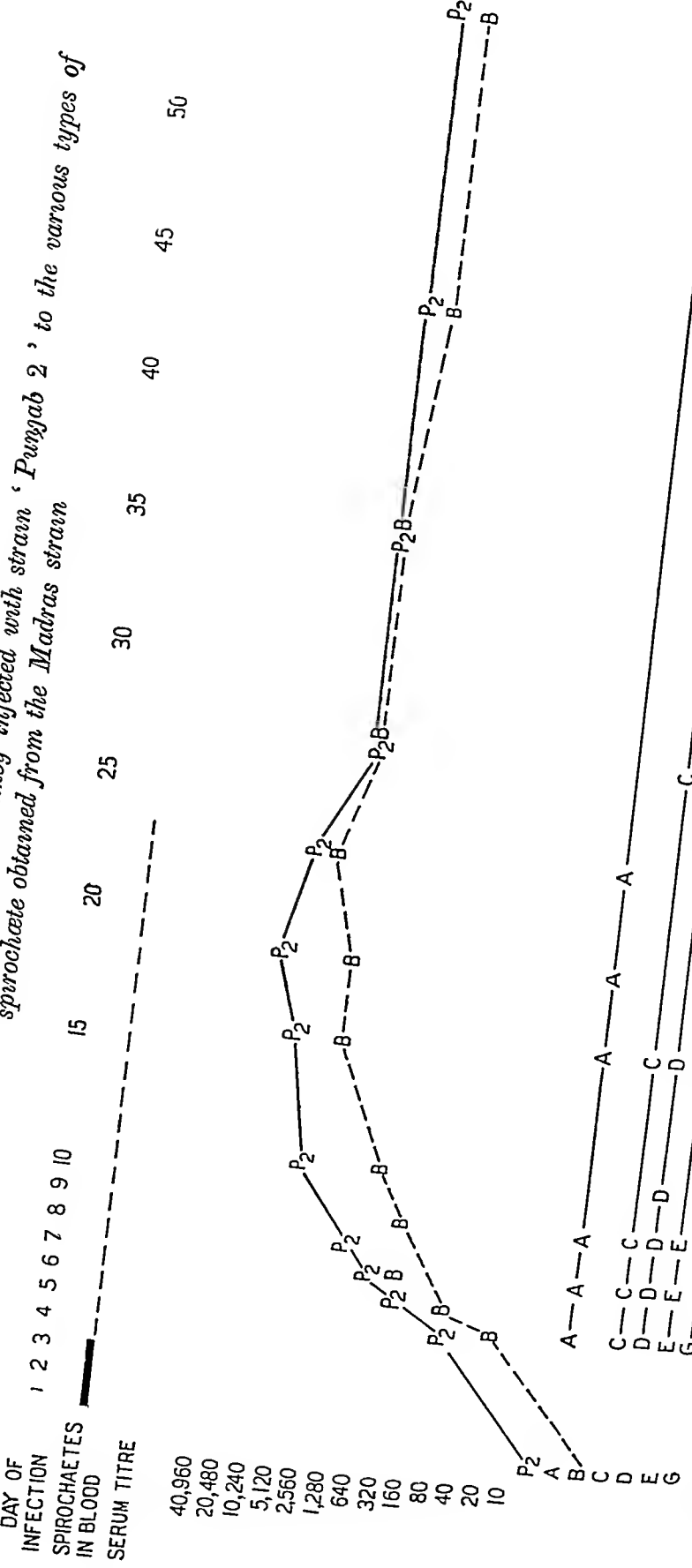
Monkey No 192 was infected with 'Punjab 2' human strain   It showed spirochætes in the blood for three days   There was no relapse   Samples of serum taken at various periods were tested against the Madras strain types ('A', 'B', 'C', 'D', 'E', 'G')   and the infecting strain 'Punjab 2'.   Identical curves for type 'B' and 'P₂' were produced   No reaction to any of the other types was obtained   The reaction to type 'B', was still present in a 1/20 dilution on the 200th day

CHART 3

Showing the agglutinin content of 132 sera from patients suffering from relapsing fever on the Nilgiri Hills, taken at various stages of the disease, tested against the 'A' type, and, in a few cases, the 'B' type of spirochæte also
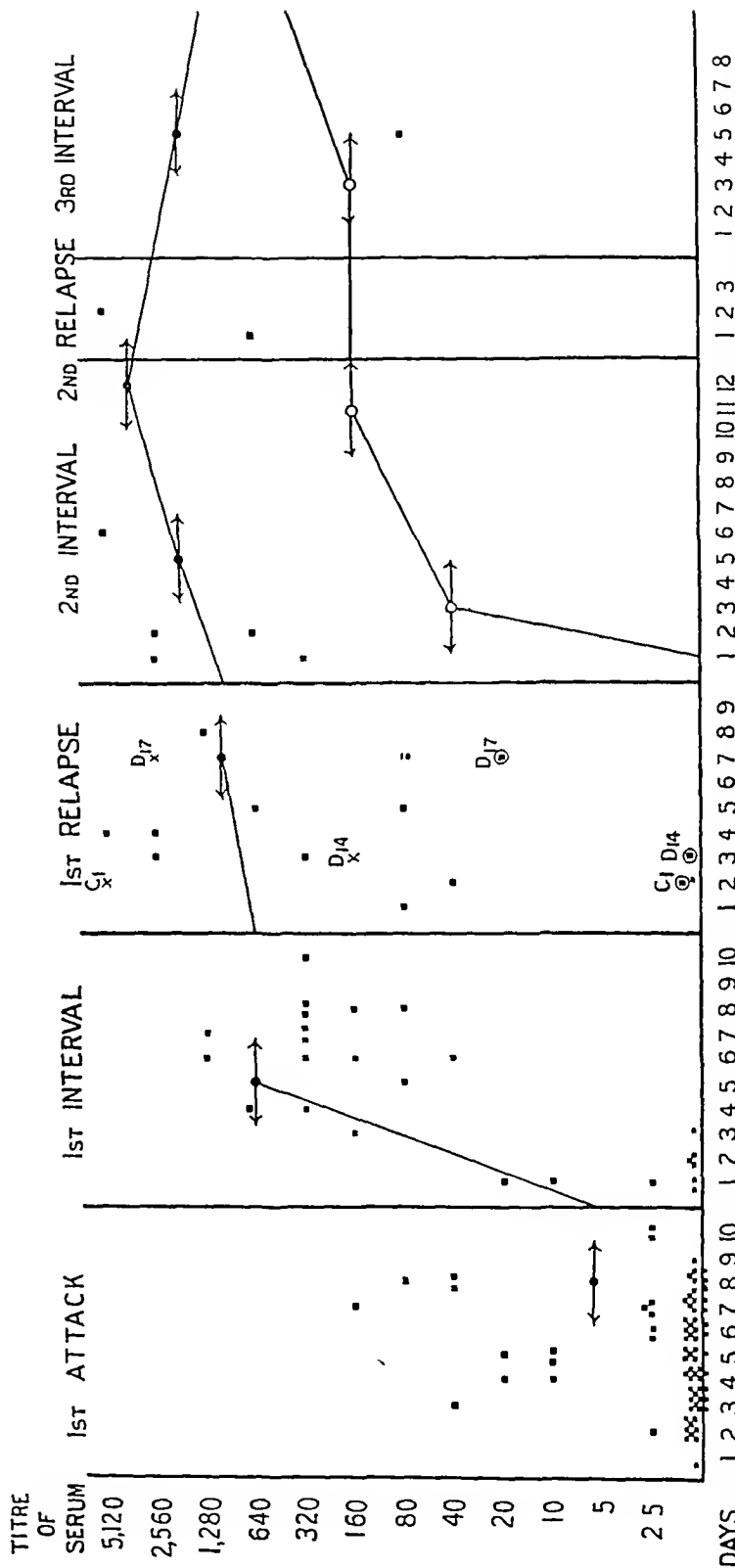
Note.—The three sera tested with the 'A' and 'B' types of spirochæte were C1, D14, and D17. In these cases the result with the 'A' type is marked ⊙ and with the 'B' type ✕

The monkey's curves for both types of spirochæte are represented by continuous lines, the black dots represent the reactions of the human sera to the 'first attack' spirochæte (type 'A') and the × the reactions to the 'relapse' spirochæte (type 'B')

The majority of the sera tested during the first attack are, as might be expected, negative. The few positive reactions call for some explanation. The smaller titres probably indicate the rise in anti-bodies occurring at the end of attacks of shorter duration. It is unlikely, however, that these reactions (at this stage when spirochætes are present in the blood) would reach titres of 1/80 or 1/160 unless some change from the type originally causing the attack had occurred. It is possible that the patients to whom these sera belonged gave wrong information as to the period of the disease at which the sample was taken and that they were, in reality, in the relapse stage, the anti-bodies present being due to a previous attack, or, alternately, these cases may be examples, similar to those recorded in our last paper (Cunningham *et al*, 1934, p 116) where the type of spirochæte causing the first attack has died out and has been succeeded by another type which of course is not influenced by the anti-bodies to the original type present in the serum

The majority of the sera taken during the first interval and subsequent stages react to the 'first attack' (type 'A') spirochæte, showing that this type was very frequently involved in the primary attack in this particular epidemic. Thirteen sera, found negative during the first interval and 1st relapse, are of special significance, for they indicate the occurrence of initial infections with the 'relapse' ('B' type) spirochæte in nature. Two such sera, tested with the 'relapse' ('B' type) spirochæte also, show that this contention is correct for they give well-marked reactions with this type of spirochæte (C1 . 1/5120, D17 1/2560), a third (D14) gives a reaction which is less definite and its connection with the disease in this particular case may, therefore, be capable of a different explanation. The serum of D17 also reacts to a certain extent to the 'A' spirochæte. This serum was taken at the end of the relapse and the reaction probably indicates the beginning of a rise to the spirochæte causing the relapse, in this case the 'A' type

Turning once more to the cases which gave positive reactions with the 'first attack' ('A' type) spirochæte, while the majority follow fairly closely the agglutination curve produced in monkeys primarily infected with this type, there are several sera, notably five, taken between the fifth and eighth days of the first interval, and three between the first and fifth days of the first relapse, which show lower titres than might be reasonably expected for the stage of the disease at which they were taken. We drew attention to these reactions in our earlier paper (Cunningham, 1925) and considered at that time that they might have some significance in the causation of second relapses. Our later work shows, we think, that these low titres can best be explained on the assumption that they are the result of secondary, and frequently latent, attacks which occur fairly commonly in experimental infections (Cunningham *et al*, 1934, p 113). Under these conditions the primary attack was due to one of the other types of spirochæte, the 'A' type assuming a minor rôle as far as these particular cases were concerned

The result recorded with D14, referred to above, and the low titre (1/80) obtained with the single sample of serum taken on the fifth day of the third interval, are both capable of the same explanation. In the latter case the three previous

attacks must have been due to three different types of spirochæte, none of which were the ' A ' type

In our first paper (Cunningham, 1925) we concluded that these results indicated a close similarity between the mechanism of infection in nature and that which we had observed under experimental conditions   Our later work simply corroborates this view by offering a satisfactory explanation of certain observations whose meaning was not obvious at that early stage in the investigation   It is of interest to note also that the ' A ' spirochæte was evidently the predominant type in this epidemic, a fact which supports our view that the ' A ' and the ' B ' types are the basic types, the remainder being of a subsidiary nature

(b) *Punjab series* —The sera referred to in this series were obtained from patients suffering from the disease in various parts of the Punjab   These cases were sporadic, or at least occurred in very small, isolated outbreaks, and in no way resembled the epidemic in South India from which the first series of sera was obtained

Information with regard to these small outbreaks was first obtained from the Public Health Department, and the cases were investigated on the spot by a travelling unit despatched from the headquarters of the investigation, assisted by the District Health Officer   Three such outbreaks were visited in Multan, Hazro and Dera Ghazi Khan   In the first two, patients were seen in the active stage of the disease, and strains of spirochæte, which have already been described ('P_1' and 'P_2' which proved to belong to the ' A ' and ' B ' types, from Multan, and a ' C ' type from the Hazro case), were isolated by sub-inoculation into animals   Samples of sera were obtained from all patients who were said to have suffered from the disease and these were sent to headquarters

The preliminary details given in each case with regard to the number of attacks of fever and the length of each attack leave little doubt that each of these outbreaks was in reality due to the ordinary ' louse-borne ' type of relapsing fever   The dirty habits of the people from whom the cases were derived and their high rate of infestation with lice corroborate this view

The results, together with all other relevant details, are given in Table I Eighteen cases were examined in all, nine from Multan, six from Hazro and three from Dera Ghazi Khan   The small amount of detail available in one of the cases belonging to the last named series (P R) makes it difficult to establish the correctness of the diagnosis, but the case has been included for reasons which will appear later

With the exception of three cases (Multan Nos 3 and 4, and Hazro No 2) where the sample of serum was obtained during the first interval, the patients were first seen at varying periods after the end of the relapse (from 6 to 118 days) when there were no symptoms and almost certainly after the infection had come to an end   For this reason the presence of spirochætes in the blood could not be verified in the majority of cases   The results of the serum examination, however, show that spirochætes similar to one or other of the types obtained from the Madras strain were responsible for the attacks in these cases also   In the majority of cases the titres to the ' A ' and ' B '

TABLE I

Giving the results of agglutination tests carried out with the various types of spirochates derived from the Madras strain and sera obtained from patients suffering from relapsing fever in the Multan, Hazro and Dera Ghazi Khan districts of the Punjab, India

Serial number.	Initials.	Age	Sex	Number of attacks	First attack	Relapse	Stage of disease	Number of days since last attack	A	B	C	D	E	F	G
					Length in days of		**Blood sample taken**		**Titre of agglutination to spirochaetal types**						
MULTAN															
1	G R	10	M	2	7	2	2 I	12	10,240	10,240	10	:			:
2	M S	10	M	2	7	2	2 I	11	2,560	640	640			:	:
3	M	25	F	1	7	1	1 I	6	5,120	1,280	2,560	80		·	·
4	A K	20	F	1	7	1	1 I	33	5,120	5,120	10,240	80		·	·
5	W B	33	M	2	9	9	2 I	118	5,120	160	640		·	·	·
6	G H	19	M	2	8	8	2 I	33	10,240	10,240	640			·	·
7	W. B	19	M	2	11	7	2 I	29	2,560	2,560	160		·	·	:

8	W B	19	M	2	9	7	2 I	32	640	1,280	640	320	
0	M	25	F	2	7	4	2 I	51	320	320	640	640	

HAZRO

1	A K	15	M	2	8	5	2 I	20	10,240	2,560	160		
2	H K	32	M	1	8	·	1 I	4		640	640		
3	A K	55	M	2	8	5	2 I	10	2,560	320	640	40	
4	M K	15	M	2	8	4	2 I	1	20,480	80			
5	M K	30	M	2	8	4	2 I	12	640	2,560	5,120		
6	U K	25	M	2	10	7	2 I	12	5,120	5,120	2,560		

DERA GHAZI KHAN

1	P R	60	M	?	?	-	I	60					
2	A R	29	M	2	6	4	2 I	6	5,120	1,280	640	640	
3	I B	28	M	2	5	4	2 I	6	10,240	1,280	1,280	1,280	640

Note.—'1 I' and '2 I' in column 8 refer to the first and second intervals

types stand highest, showing that these two types have, once more, been the ones principally concerned in the primary attack and the relapse   The existence of subsidiary types is also evident from the positive reactions to a third, and sometimes a fourth, type of spirochæte in the greater number of instances   In fact, the serum response in these human cases bears a very close resemblance to that shown to be present at similar stages of the disease in the experimental animal (Cunningham *et al*, 1934, p 112) and also proves that there is little or no difference between the louse-borne infections in Northern and Southern India

(c) *North-West Frontier series*—The cases which make up this group occurred in three different areas in the North-West Frontier Province—Parachinar (in the Kurram Valley), Drosh (south of Chitral) and Quetta (Baluchistan)   They occurred amongst the Military units or the followers attached to them

The natural history of the disease in these cases is so different from that of the louse-borne type with which we have been familiar up to the present, that a preliminary account of the special features of the disease as it occurs in these areas will be necessary before recording the results of our serological tests

Our attention was first drawn to relapsing fever in the Kurram area by Major H J H Symons, M C , I M S (1926), the Agency Surgeon at that time, who informed us that the disease had been known in the valley for many years, the number of cases reported amongst the militia varying from three or four to 28 in different years   He gives the following description of its clinical features —

' The type of relapsing fever met with in the Kurram is, as a general rule, not severe, in that the fever rarely lasts more than twelve hours or so, and, for this reason, its diagnosis, or at least early diagnosis, is difficult, as sepoys, as a rule, do not come to the hospital until at least an hour or so after the beginning of the attack, and unless the blood smear is taken at the very beginning of the pyrexia, spirilla are very rarely, if ever, found '

' The attack may or may not start with a rigor , the absence of the rigor has been found to be the rule rather than the exception   In some cases a temperature of 103 5°F has been recorded and in some only 99°F or 100°F   The onset is accompanied by pains in the back and limbs and headache of varying severity   In fact at the start there is virtually no great difference between the symptoms of this disease and those of sand-fly fever   No enlargement of the spleen was detected in any case   No case was complicated with jaundice, nor did any case show gastric or intestinal symptoms '

' This type of relapsing fever is most often very resistant to treatment, running up to six or eight relapses   In one case the relapse lasted for three days, and, in a few instances, for two days, but the usual case rarely lasted for 24 hours   Relapses which occurred in cases not diagnosed on admission occurred on any day from the third to the thirteenth '*.

Major Symons also refers to an outbreak where ' all the servants residing in the servants' quarters of a certain bungalow developed relapsing fever and showed

* Some of the charts received from Drosh show longer intervals

bites of some biggish bug or louse, but no bugs or lice could be found on their clothing or in their charpoys '*

Certain of the temperature charts kindly supplied by Major Symons illustrate well the salient points of the attacks and are reproduced in Charts 1 to 3 below Blood smears, case records and temperature charts forwarded at a later date from Drosh (Charts 4 to 6 below) through the kind agency of Major J M Macfie, R A M C (1928), the D A D P , Peshawar, at that time, showed that the same variety of the disease was in evidence there as the result of bites of some unidentified insect, and certain cases reported from Quetta by Colonel A A McNeight, I M S (1927), also proved to be of the same nature

From examination of the temperature charts and case records there is little doubt that these cases are examples of the type of tick fever originally described by Browse (1912), in Quetta, and later by Smith and Graham (1913) in Chitral   From a further comparison with the descriptions of the Persian type of relapsing fever given by Dschunkowsky (1913), Harold (1920) and Wright and Harold (1920) there seems to be no reason to doubt that the tick-borne fever in all these different areas is really a single entity, a view which receives support from the work of more recent observers who report the Persian type of the disease as occurring in Bokhara and Turkistan (Starobynsky, 1922 , Samsonov, 1926 , Pickoul, 1928 , Doubrowine, 1928 , Tschirejkin, 1930 , Pavlovskii, 1930 , Kassirsky, 1933) and in the Pamirs bordering on India and Afghanistan (Katz, 1930)

Fry (1920), Harold (*loc cit*), Sinton (1921) and others point out, however, that the louse-borne type of the disease is also prevalent in these areas   The two varieties of the disease may, therefore, run concurrently in the same area, the former capable of taking on epidemic form, the latter being more endemic and attached to definite localities and even buildings , an observation which is also true of the disease in other parts of the world

Nicolle and Anderson (1928), on the strength of experiments carried out with *O papillipes* received from Bokhara, hold that the spirochæte responsible for the tick-type of fever in this region differs from those previously described (chiefly in the susceptibility of guinea-pigs to infection), and have named it *Sp sogdianum* Pickoul (*loc cit*) has named the same spirochæte *Sp ustelistanica*   There appears to be general agreement that *O papillipes* is the natural vector of this variety of the disease (Nicolle and Anderson, *loc cit* , Moskwin, 1929 , Kritschenski and Dvolaitskaya-Barischewa, 1931 , Pavlovskii, *loc cit*) although other authorities have incriminated *O tholozani* (Dschunkowsky, *loc cit* , Starobynsky, *loc cit* , Samsonov, *loc cit* , Marzinowsky, 1927)

We were never in close enough contact with these cases to be able to obtain sub-inoculations into animals from them   The officers concerned, however, were kind enough to supply us with blood smears and samples of serum taken from patients at various stages of the disease which proved that we were undoubtedly dealing with cases of relapsing fever and enabled us to institute a comparison between the two varieties of the disease with very interesting results

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* Certain ticks from the roof of this house were sent to us for identification   These proved to be *Argas persicus*   Prolonged attempts to make these insects feed on monkeys proved unsuccessful, although they readily fed within ten minutes when placed on fowls

The results of the examination of these patients' sera against the different types of spirochæte derived from our louse-borne Madras and Punjab strains are

TEMPERATURE CHART 6

Name A. D. _Drosh.   *Age* 33.

Day of disease.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Pulse m	100	96	76	74	74	64	68	64	78	94	70	70	70	72	70	68	72	72	76	76
Pulse e	106	100	102	74	76	66	82	76	102	86	68	74	72	66	70	70	72	72	72	74
Resp m	26	23	22	20	21	17	17	18	18	22	18	19	17	18	18	17	18	18	18	18
Resp e	24	26	24	21	18	18	22	20	32	23	18	20	18	17	17	18	18	18	18	
Motions																				
Urine ozs																				
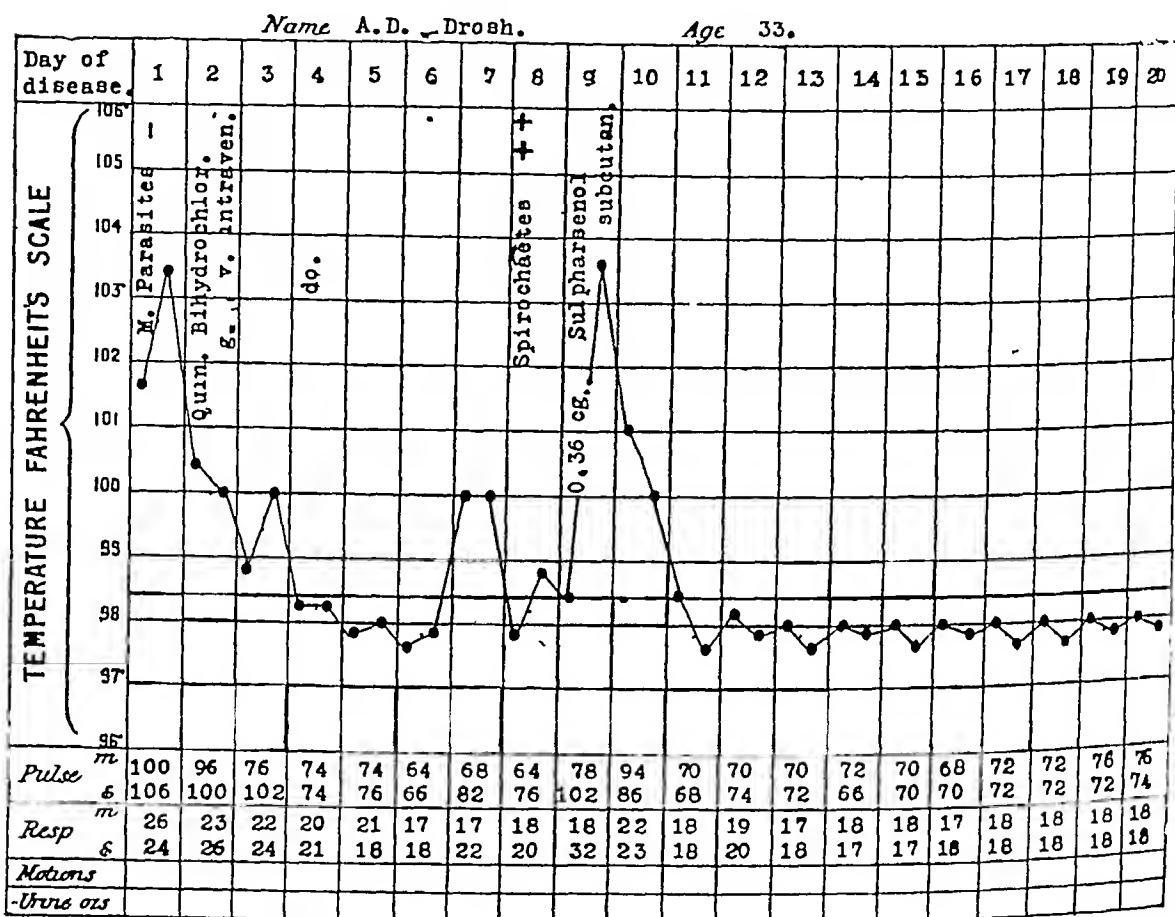
set forth in Tables II, III and IV Twenty-nine cases were examined in all 13 from the Kurram (Table II), 10 from Drosh (Table III), and six from Quetta (Table IV) In a considerable number also, more than one sample of serum was received from the same case, so that it was possible to verify earlier results by examinations which were carried out at a later stage As matters turned out it was fortunate that this was possible, for the picture presented by these cases was quite different from that shown in the previous tables which dealt with outbreaks of the louse-borne types of the disease in other parts of India

No less than two-thirds of the cases in the present groups (19 out of 29) failed to show any reaction whatsoever with any of the types of spirochæte in our possession, although the samples of serum were taken at a stage of the disease when a positive result might confidently have been expected in the louse-borne type The cases giving negative results were found in all three areas as follows the Kurram, 7 out of 13 , Drosh, 9 out of 10 , and Quetta, 3 out of 6 Of the

TABLE II

Giving the results of agglutination tests carried out with the various types of spirochetes derived from the Madras strain and sera from patients suffering from relapsing fever on the Kurram Valley on the North-West Frontier of India

Serial number	Initials	Age	Sex	Number of attacks	Length of each attack in days and presence of spirochaete in blood		Blood sample taken		Titre of agglutination to spirochaetal types								
							Stage of disease	Number of days since last attack	A	B	C	D	E	F	G	H	I
1	M K	28	M	2?	10? 1*	-	2 I	5			10,240	80					
					1*	+	2 I	9			40,960	160					
							1 I	21			10,240	40					
2	H G	26	M	4	1* 1*	+	4 I	11			1,280	160					
					? 1*	+	2 I	26			2,560						
3	P L	30	M	2?	-	+	1 I	30			640						
4	R W	25	M	3?	1* 1*	+	3 I	6				?					
5	S S	19	M	4	? ?	+	5 I	13		80	160						
					1 1	+	5 I	70		?	160						
6	S G	19	M	2	1	-	3 I	5							320		
						-	3 I	10									
7	M S	—	M	3	1* 1	+	2 I	3									
					1 -	-	3 I	10									
8	D K	22	M	1	1* 1*	+	1 I	10									
9	A G	23	M	1	2* 2*	+	1 I	12			20 ?						
10	R S	19	M	4	2* 1*	+	1 I	94									
					2* 1*	-	1 I	11									
11	J K	21	M	3	- -	1*	4 I	72									
12	L J	26	M	5	2 1	1	3 I	47									
13	W K	20	M	7	2* 1* 1* 1* 1* -	+	5 I	14									

Note.—In column 7 the sign 1 I 2 I, etc, with the numeral before it refers to the first or second interval, the numeral following it giving the exact number of days since the last day of the fever of that particular attack

The sign * denotes that one or other of the arsenical preparations (Stabilarsan, Sulpharsenol or Stovarsol) was given at this period of the disease In certain cases the number of attacks was cut short by this means but in others the disease persisted in spite of the treatment

10 positive cases only four reacted in dilutions of over 1/1000, in six cases the sera reacted with two or more types of spirochæte Positive reactions were obtained with the various types as follows —

Types of spiro-chæte	A	B	C	D	E	F	G	H	I
Number of cases	2	3	8	2			2		

a distribution which is at variance with the findings already recorded for other areas in India where the 'A' and the 'B' spirochætes were the types most commonly involved

Turning to the individual cases, the nature of the reaction recorded with the serum of M K (Table II, Kurram Valley No 1) leaves little doubt that this case was an example of the louse-borne type of the disease, and the length of the first attack (10 days), combined with the fact that there was only a single relapse and that the patient contracted the disease while on leave in India, all corroborate this point of view It is possible also that A D (Table III, Diosh No 1), N K and W S (Table IV, Quetta Nos 2 and 3) may be examples of the same kind but the supporting evidence in these cases is not so definite as it is in the case of M K

On the other hand, definite positive reactions have been obtained with cases which, according to the clinical picture, undoubtedly belong to the tick-borne variety of the disease Examples of these are H G (Table II, Kurram Valley No 2) who showed four short attacks in only two of which the spirochæte was found and whose serum reacted to the 'C' and 'D' types of spirochæte in dilutions of 1/2560 and 1/640 respectively, R N (Table II, Kurram Valley No 4) who had three or more attacks of the same type (spirochæte found on two occasions only) and whose serum reacted with the 'G' type of spirochæte (1/320), and P L (Table II, Kurram Valley No 3) whose serum gave a positive reaction to the 'C' and 'B' types of spirochæte (1/640 and 1/80 respectively)

The remaining three positive cases, also belonging clinically to the tick-borne variety, gave reactions to the 'C' type of spirochæte alone and in strengths which lie between a dilution of 1/640 (M K, Table IV, Quetta No 1) and a mere trace of a reaction, 1/20 (R S, Table II, Kurram Valley No 10)

It would appear, then, from the results obtained with this group as a whole that both varieties of the disease are probably represented on the North-West Frontier, the cases carried by the louse giving the same type of reaction as those already described for other regions, the examples of the tick-borne variety, on the other hand, giving a poor or no immunological response to our louse-borne types of spirochæte

Our results show, we think, that the spirochæte responsible for the tick-borne form of the disease in this area is either a different serological entity or else is rendered incapable of stimulating the formation of the anti-bodies which play such an important part in bringing the attacks of the louse-borne variety to an end The short-ness of the attacks and the scarcity of the spirochætes in the blood are factors which

favour the latter view, but the presence of positive reactions in certain of our cases shows that under certain conditions anti-body formation is possible

Taking these latter cases into consideration we are inclined to the view that there may be some serological connection between the Frontier strain of spirochæte and our louse-borne types and that the spirochæte may have been modified in some way by passage through a different insect vector   We were unfortunately not in close enough touch with the cases themselves to investigate this hypothesis more precisely by means of experiments with the strains of spirochæte responsible for the two types of the disease

The formation of anti-bodies by the tick-borne varieties of spirochæte is reported by many authors (Brussin, 1925, 1926 , Brussin and Rogowa, 1927 , Jackimow, 1928, 1929 , Gori, 1928)   At the same time other observers have not been able to demonstrate their presence (Gray, 1928, 1929 , Plaut and Grabow, 1930)

One of us (J C) had the further opportunity of testing our louse-borne type sera and the sera derived from some of the cases of the tick-borne fever in the Kurram with three other strains of spirochæte obtained from different laboratories in Europe *   Two of these produced scanty but persistent infections in mice similar to those usually described for *Sp duttoni*, the other produced heavy progressive infections similar to those observed in infections with *Sp carteri* in India

In no case was a definite positive reaction obtained with either the ' louse-borne ' sera or the Kurram Valley patients' sera, although a trace was observed with the ' Berlin ' strain and our strains ' G ' and ' D '   We were forced to the conclusion, therefore, that the European strains tested and the Indian types of spirochæte did not contain any antigen in common   As far as the Edinburgh strain (*Sp duttoni*) was concerned, however, Gray (1928, 1929) was unable to demonstrate the presence of anti-bodies of any kind in his own blood or in the blood of experimental animals, after infection with this organism   It may be, therefore, that certain strains, of which this organism is one, neither produce nor react with spirochætal anti-bodies

Whether or not any definite serological relationship will ultimately be shown to be present between the organisms responsible for the louse-borne and tick-borne varieties of relapsing fever in India, there seems to be little doubt that the ' mechanism ' of the two diseases is quite distinct   Some process seems to be at work in the tick-borne variety which checks the ever-increasing multiplication of the spirochætes, which, in its turn, causes the prolonged febrile attacks with the formation of anti-bodies on a grand scale   Whether this process is inherent in the strain of spirochæte or is due to some agency on the part of the host, either insect or human, is a question which must await further investigation for an answer

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* Two of the above three strains were obtained through the kind offices of Colonel S P James, F R S , I M S (retd), and Professor Nocht of Hamburg, from Professor Kolle of the Institut für Experimentelle Therapie, Frankfurt a M and Professor Kroo, Robert Koch Institut, Berlin   The first of these was labelled ' Russian Recurrens, *Sp obermeieri* ', the second, ' *Sp obermeieri* '   The third was a strain of *Sp duttoni* lent by Professor T J Mackie of Edinburgh University and was originally obtained from the London School of Tropical Medicine   This strain was used by Gray in his work on this subject (1928, 1929)   No information of the ultimate source of any of these strains was obtainable

One further point requires mention   Attention has already been directed to one of the Dera Ghazi Khan group of cases (P R No 1) whose serum failed to react with any of our 'louse-borne' types   The reactions obtained in this case thus resemble those recorded with the tick-borne cases occurring in the trans-frontier stations   Unfortunately full details with regard to the number and type of the attacks were not available in this case, but blood films taken early in the attack showed spirochætes, while those taken later in the day were reported negative   This fleeting appearance of spirochætes is significant, and, taken in conjunction with the serological reactions, makes it probable that we are dealing with an example of the tick-borne variety of the disease   If this is the case then this form of the disease must occur in more easterly districts in India than has been recorded hitherto and a careful watch for such cases should be kept in future with a view to investigating the extent of the endemic area and studying further the epidemiology of this type of the disease

SUMMARY

1   A comparison of the disease as it occurs in experimental animals and in human beings has been made by (1) the examination of spirochætes derived from different natural human infections, and (2) the investigation of the agglutinin content of the serum of patients at different stages of the disease to the various louse-borne types of spirochæte which have come to light as the result of an investigation into experimental infections in animals

2   Spirochætes isolated from three separate human examples of the louse-borne type of the disease in the Punjab proved to be identical with three of the types derived from the Madras strain (strains 'Punjab 1' with type 'A', 'Punjab 2' with type 'B', and 'Hazro' with type 'C')

3   The human sera tested were derived from epidemic and sporadic outbreaks of the disease in various parts of India as follows —

   (a) Samples taken from 132 cases obtained from an epidemic of the disease in the Nilgiri Hills in the Madras Presidency in 1924

   (b) Eighteen samples from three sporadic outbreaks occurring in the Punjab ,—Multan, Hazro and Dera Ghazi Khan

   (c) Twenty-nine samples from three areas in the North-West Frontier ,— the Kurram Valley, Drosh (near Chitral), and Quetta

4   The majority of the sera from the outbreak in the Nilgiri Hills were tested against the 'A' type of spirochæte only , a few against the 'B' type   The results of the tests were consistent with the view that the majority of the infections in this epidemic were caused by the 'A' and 'B' types and that the course of anti-body formation followed closely that described as occurring in experimental animals

5   Similar conclusions could be drawn from the tests carried out with the samples of sera obtained from Multan, Hazro, and Dera Ghazi Khan (with the exception of one case) in the Punjab, and further that the nature of louse-borne infections in the North of India was similar to those encountered in the Madras Presidency.

6   Evidence of the existence of the Persian variety of tick-borne relapsing fever, as well as the louse-borne type, was obtained from a study of the cases which occurred in the three areas in the North-West Frontier   The numerous short

attacks, the scarcity of spirochætes in the blood and the resistance of the infection to treatment—all typical features of this variety of the disease elsewhere—were noted by the medical officers in charge of the cases

7 The serum of the majority of cases in this group failed to show any reaction with any of the louse-borne types of spirochæte already isolated, although repeated tests were carried out at suitable periods during the disease   Certain of the cases which gave positive reactions were, in all probability, examples of the louse-borne variety , but reactions were also obtained in certain cases in which the clinical picture was undoubtedly that of the tick-borne type

8 In these cases the largest number of positive reactions occurred with the 'C' type of spirochæte   a distribution which is at variance with the findings already recorded in other areas in India where the louse-borne type of the disease is prevalent

9 The 'natural history' of the two varieties of the disease is quite distinct, whatever the serological relationship between the spirochætes responsible for the two types of the disease may be

10 Attention is drawn to the possibility of one of the cases which occurred in Dera Ghazi Khan being in reality an example of the tick-borne variety of relapsing fever   If such was the case, then this form of the disease occurs in more easterly districts in India than has been recorded hitherto

11 Reference is made to further tests carried out with the Indian 'louse-borne' type sera and certain 'tick-borne' sera (from patients who had suffered from the disease in the Kurram Valley) and strains of spirochæte obtained from different laboratories in Europe   No positive reactions were recorded in any of these tests

CONCLUSIONS

1 The types of *Sp carteri* appearing in louse-borne epidemics of relapsing fever in North and South India are identical

2 The principal serological types involved in these outbreaks are those previously described as types 'A' and 'B'

3 Anti-body formation in human cases of the louse-borne variety of the disease follows the same course as that already described in the experimental animal

4 The Persian (tick-borne) variety of relapsing fever exists on the North-West Frontier of India   The 'natural history' of these two varieties of the disease (louse-borne and tick-borne) is quite distinct

5 Some serological affinity between the spirochætes causing the two varieties of the disease appears probable, but its extent is doubtful   Most of the sera derived from patients on the North-West Frontier failed to react with any of the louse-borne types of spirochæte already described   Where reactions did occur, however, the 'C' type was the one most frequently involved

6 Sera obtained from louse-borne and tick-borne infections in India failed to agglutinate certain strains of spirochæte obtained from various European laboratories

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Note —References marked * were consulted in the *Tropical Diseases Bulletin* only and not in the original

STUDIES ON THE ANTIGENIC STRUCTURE OF *VIBRIO CHOLERÆ*

Part VIII.

THE SPECIFIC CARBOHYDRATE CONTENT AND SEROLOGY OF THE ACID-SOLUBLE FRACTIONS

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WE have previously reported the results obtained from the study of the three fractions into which the vibrios can be divided by extracting them with 0 025 N and 0 05 N HCl in alcohol (Linton and Mitra, 1934)   The first fraction, which we designated the 'A' fraction, appeared to have a characteristic constitution quite distinct from that of the whole vibrio and also from that of the second 'B' fraction, and the Residue left after removing 'A' and 'B'   This difference was found in its Hausmann nitrogen analysis, in its 'racemization' curve in dilute alkali, and in its specific rotation

We have continued the study of these fractions in a representative group of vibrios (Table I), and are presenting in this paper some further data on their distribution in the vibrios, on the distribution of the specific carbohydrates in the fractions themselves and the relation between this distribution and agglutinability, and on some cross-reactions with anti-sera against the whole vibrios and against these separate constituents

METHODS

In our experience, the minimum amount of growth necessary for satisfactory analyses of the kind reported here is that obtained on the equivalent of 150 Roux flasks incubated for 48 hours   In most of our experiments this amount was exceeded, and in some of them, doubled   The vibrio growth was washed off in

distilled water to which phenol had been added to make a 1 per cent solution, and the whole mass put through the Sharples centrifuge, taken up in distilled water and the process repeated until the wash water, after boiling it with 3 per cent H_2SO_4 to hydrolyse any agar present, no longer reduced Benedict's solution   It is essential that the washing and testing of the supernatant fluid be carefully carried out, and the process continued until a negative result is obtained, as otherwise an appreciable quantity of agar and reducing substance from it may be carried over, and thus invalidate the succeeding experiments on the reducing substances of the vibrios themselves   The final washings were taken up in 1 litre of 95 per cent alcohol and heated for one-half hour at 50°C   After centrifuging, the 'A' and 'B' substances were extracted according to the following scheme —

> 'A' *substance*—1st extraction 975 c c absolute alcohol and 25 c c N/1 HCl, to make a 0 025 N solution
>
> Heated for one and one-half hours in the water-bath at 55°C , with constant stirring
>
> Second extraction identical in every way with the first

After each extraction the mass was cooled and run through the Sharples centrifuge and the slightly turbid fluid was put through a Seitz filter, using an E-K disc   The two water-clear extracts were united, concentrated *in vacuo* to 150 c c to 200 c c , and precipitated with three volumes of ether   After standing overnight in the ice-box, the light-brown, granular precipitate was collected and dried in an air-oven at 50°C   'A' substance is extremely hygroscopic and the larger amounts, such as we have worked with here, do not dry satisfactorily at 37°C , although we were able to use this temperature in our previous work with smaller amounts

> 'B' *substance* —After 'A' had been extracted, the vibrios were taken up in the following solution —
>
> Seven hundred and fifty c c absolute alcohol, 200 c c normal saline, and 50 c c N/1 HCl, to make a 0 05 N solution
>
> Second extraction made in exactly the same way
>
> Third extraction 375 c c absolute alcohol, 100 c c normal saline, and 25 c c N/1 HCl

In the case of each of these extractions the vibrio suspension was boiled for 15 minutes (b p 82°C)   After cooling and centrifuging, the extracts were added together, put through a Seitz filter (E-K disc) and the clear brown fluid brought to the point of maximum precipitation by the addition, slowly and with constant stirring, of strong NaOH   The maximum precipitation of 'B' substance was found to occur quite sharply at a point just slightly acid to litmus   After standing overnight in the ice-box the 'B' substance was collected and dried at 50°C in the air-oven   The precipitate of 'B' is floccular, light and bulky, while that of 'A' is granular and collects at the bottom of the cylinder after a few hours' standing in the cold

After the extraction of 'A' and 'B' the Residue was dried at 50°C , or more usually at 37°C , and weighed   From it the specific carbohydrate was then extracted as follows   The dried mass was powdered and stirred into 100 c c to 400 c c (depending on the weight) of 4 per cent NaOH, placed at 37°C for 2 hours and stirred frequently   At the end of this period practically all of the material

was in solution and this was taken, brought just to the alkaline side of litmus with HCl, and enough absolute alcohol added to cause complete precipitation The amount of alcohol necessary for this purpose varied between 3 and 10 volumes with different strains of vibrios After standing for an hour or two, the precipitate was centrifuged off, taken up in distilled water and stirred thoroughly A few drops of concentrated HCl were then added until the precipitation of the protein was complete After centrifuging, the clear supernatant was put aside and the protein again taken up in water, made alkaline to facilitate solution of the carbohydrate, reprecipitated with acid and again centrifuged The two supernatants were then added together, made slightly alkaline and precipitated with 1 5 volumes of absolute alcohol Under these conditions the specific carbohydrate usually flocculates at once, and may be collected, washed till free from alkali and dried within 3 or 4 hours If allowed to stand for much over 24 hours a varying amount of inorganic material also precipitates

The dried 'A' and 'B' extracts, and the specific carbohydrate extracted from the Residue, were finely powdered, taken up in 10 c c or more of 3 per cent H_2SO_4, and hydrolysed in the boiling water-bath In the earlier part of the work the percentage hydrolysis was followed at intervals up to 6 hours, and it was found that after two and one-half hours the amount of reducing substance either showed no change or was somewhat decreased, and this period was accordingly adopted uniformly in the later experiments

RESULTS

Altogether 16 extractions on 10 strains of vibrio have been made The origin of these strains, the type of growth they exhibit and the groups into which they fall on the basis of their protein and carbohydrate structure are shown in Table I —

TABLE I

Data on the strains of cholera and cholera-like vibrios used

Strain	Source	Type of growth on agar	Vibrio group *
1617	Cholera	Smooth	I
Rangoon Smooth	Cholera	Smooth	I
Rangoon Rough (1)	From Rangoon Smooth	Rough	I
Rangoon Rough (2)	From Rangoon Rough (1)	Rough	III
Basrah I	Cholera	Smooth Rough	IV
2027	Cholera	Smooth	II
505	Cholera	Smooth Rough	II
W 880	Water	Smooth Rough	III
El Tor	Human, non cholera	Smooth Rough	IV
79 B	Cholera	Smooth Rough	IV

* The basis of this classification has been detailed in a paper by Linton, Mitra and Shrivastava (1934), and a more extended discussion is included in the succeeding paper of this series (Part IX) (pp 633–657, this issue)

Table II contains the quantitative results of the extractions —

TABLE II

Yields of 'A', 'B' and Residue substances from the vibrios

Strain	'A' substance, g	'B' substance, g	Residue, g	Total weight, g	Percentage 'A'	Percentage 'B'	Percentage Residue
1 1617	0 60	0 85	8 2	9 65	6 2	8 8	85 0
1617	0 20	0 75	8 6	9 55	2 0	7 8	90 2
1617 .	0 40	0 95	10 0	11 35	3 5	8 3	88 2
2 Rangoon Smooth	0 29	1 15	8 0	9 44	3 0	12 0	85 0
3 Rangoon Rough (1)	0 14	0 69	4 5	5 33	2 5	12 7	84 8
4 Rangoon Rough (2)	0 37	0 63	4 23	5 23	7 0	12 0	81 0
5 Basrah I	0 32	0 81	8 58	9 71	3 3	8 3	88 4
6 2027	0 23	0 82	3 1	4 15	5 2	20 0	74 8
2027	0 15	0 88	7 04	8 07	1 8	11 0	87 2
7 505	0 84	1 07	11 64	13 55	6 2	8 0	85 8
505	0 38	0 78	8 44	9 60	4 0	8 0	88 0
8 W 880	0 81	1 13	13 30	15 24	5 3	7 4	87 3
W 880	0 69	1 08	8 31	10 08	6 8	10 7	82 5
W 880	0 87	1 89	22 08	24 84	3 5	7 5	89 0
9 El Tor	0 26	0 64	9 82	10 72	2 4	6 0	91 6
10 79-B	1 26	1 47	14 02	16 75	7 5	8 8	83 7

The data in Table II are closely similar to those which we previously reported In any given extraction the amount of 'A' is always less than that of 'B', and at the same time we have again observed that with repeated extractions of the same strain absolutely concordant percentage results are not obtained In general the percentages reported here are somewhat higher than those found previously 'A' substance averages 4 2 per cent against 2 2 per cent and 'B' substance 9 8 per cent against 6 6 per cent In that paper our study was made on about one-tenth as much growth, and it is possible that the greater proportionate loss inherent in working with the small amounts may account for the differences observed in the percentage yields

In Table III are given the absolute amounts and percentages of reducing substances obtained after the hydrolysis of the 'A' and 'B' fractions and of the specific carbohydrate of the Residues —

TABLE III

Reducing substances in the 'A', 'B' and Residue fractions of the strains of vibrios

Strain		REDUCING SUBSTANCES IN			Total, mg	PERCENTAGE OF REDUCING SUBSTANCES IN		
		'A', mg	'B', mg	Residue, mg		'A'	'B'	Residue
1	1617	9 7	9 3	11 5	30 5	32	30	38
	*1617	5 5	11 0	7 5	24 0	22	46	32
	1617	6 0	11 0	10 0	27 0	22	41	37
2	Rangoon Smooth	4 5	17 0	3 5	25 0	18	68	14
3	Rangoon Rough (1)	4 2	40 0	35 5	79 7	5	50	45
4	Rangoon Rough (2)	7 0	39 0	20 0	66 0	11	59	30
5	Basrah I	3 0	48 0	6 0	57 0	5	84	11
6	2027	4 5	10 2	2 3	17 0	25	60	15
	2027	4 2	10 0	3 0	17 2	25	58	17
7	505	8 0	15 0	13 0	36 0	22	41	37
	505	6 5	17 0	3 0	26 5	24	64	12
8	W 880	5 6	62 0	15 0	82 6	6	75	19
	*W 880	5 3	19 0	6 2	30 5	17	62	21
	W 880	12 0	40 0	16 0	68 0	18	59	23
9	El Tor	2 5	31 0	4 0	37 5	7	83	10
10	79 B	8 0	72 0	7 0	87 0	9	83	8

* In these two instances the specific carbohydrate was not separated from the Residue, and the reducing power was determined after the hydrolysis of the whole Residue protein Comment in text

In general the data given in Table III show that, considered absolutely, 'B' fraction usually contains more of the reducing substance than either of the other fractions   In some cases (e g , Basrah I and El Tor) 'B' fraction may possess no less than four-fifths of the total reducing power of the vibrio carbohydrate   It is also clear that 'A' and 'B' together contain, in proportion to their amounts in the vibrio, much more reducing substance than the Residue does   The data in Table III have been considered in relation to the proportionate amounts of each fraction, by taking each fraction separately to represent 100 per cent of the whole vibrio and calculating the amount of reducing substance which would be present This calculation gives a figure for the reducing substances in each of the fractions in proportion to the amount of the fraction   The results are given in Table IV and diagrammatically in the Graph —

<div align="center">

TABLE IV

Reducing substances in the 'A', 'B' and Residue
fractions of various vibrios in relation
to the amount of each fraction

</div>

Strain		Fraction	Percentage of whole vibrio	Calculated amount of reducing substance in proportion to percentage of fraction
1 1617	{	'A' 'B' Residue	6 2 8 8 85 0	156 106 14
1617	{	'A' 'B' Residue	2 0 7 8 90 2	244 141 8
1617	{	'A' 'B' Residue	3 5 8 3 88 2	171 131 11
2 Rangoon Smooth	{	'A' 'B' Residue	3 0 12 0 85 0	150 139 4
3 Rangoon Rough (1)	{	'A' 'B' Residue	2 5 12 7 84 8	170 315 42
4 Rangoon Rough (2)	{	'A' 'B' Residue	7 0 12 0 81 0	99 322 25

TABLE IV—*concld*

	Strain	Fraction	Percentage of whole vibrio	Calculated amount of reducing substance in proportion to percentage of fraction
5	Basrah I	'A'	3 3	91
		'B'	8 3	571
		Residue	88 4	7
6	2027	'A'	5 2	85
		'B'	20 0	51
		Residue	74 8	3
	2027	'A'	1 8	236
		'B'	11 0	92
		Residue	87 2	3
7	505	'A'	5 2	127
		'B'	8 0	190
		Residue	85 8	15
	505	'A'	4 0	164
		'B'	8 0	209
		Residue	88 0	3
8	W 880	'A'	5 3	106
		'B'	7 4	835
		Residue	87 3	17
	W 880	'A'	6 8	78
		'B'	10 7	178
		Residue	82 5	8
	W 880	'A'	3 5	343
		'B'	7 5	526
		Residue	89 0	18
9	El Tor	'A'	2 4	100
		'B'	6 0	521
		Residue	91 6	4
10	79 B	'A'	7 5	111
		'B'	8 8	818
		Residue	83 7	8

As the data in Table IV and the Graph show, the preponderant amount of reducing substance (in proportion to the quantity of the fractions) is present in

the 'A' and 'B' fractions, which on the average have together about 45 times as much as the Residue, 'A' having 15 times and 'B' 30 times the amount The possible position of these amounts of reducing substance in the vibrios is of interest On the basis of the mode of extraction we have used it appears not unlikely, especially in the case of 'A' fraction, that the 'A' and 'B' fractions may represent the outer portions of the vibrios The demonstration of reducing substances in these

GRAPH
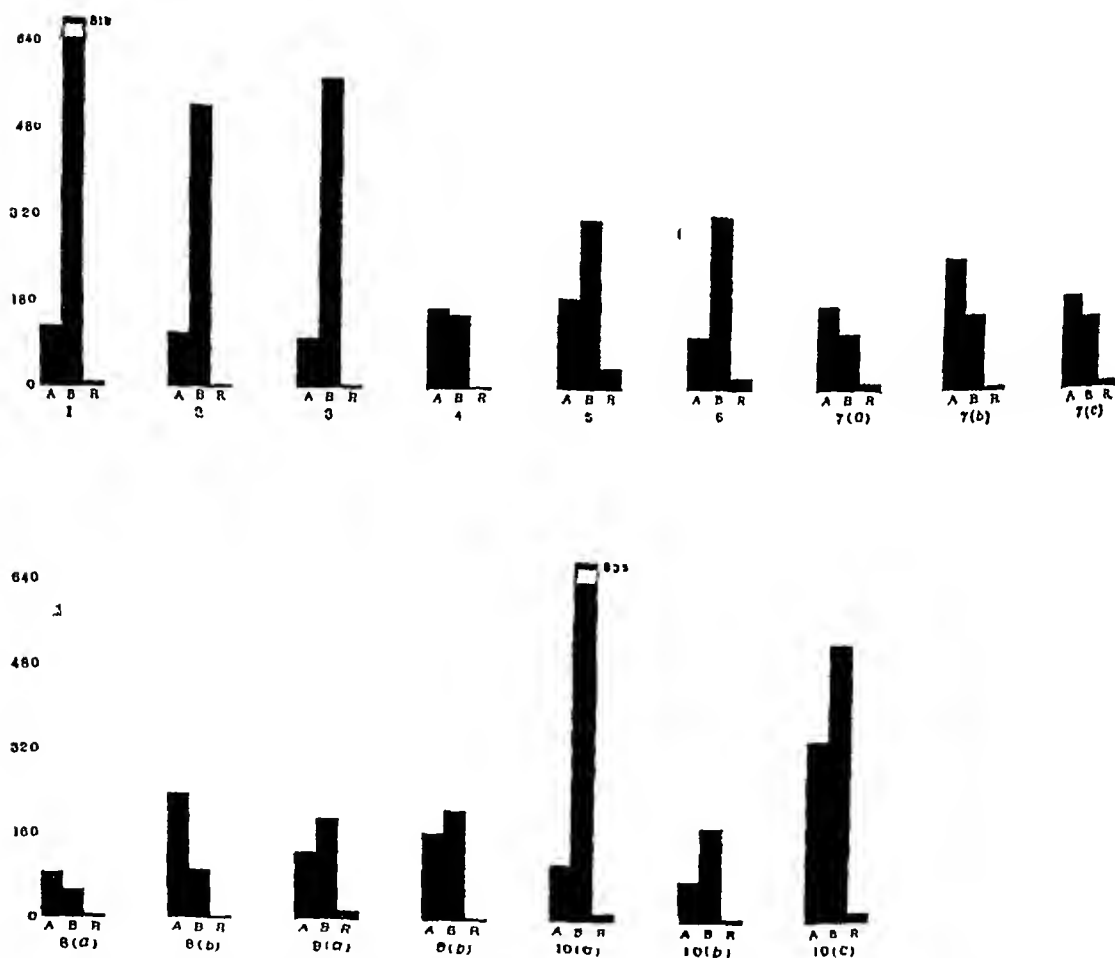
Proportionate amounts of reducing substances in the 'A', 'B' and Residue fractions of the vibrios 1, 79-B, 2, El Tor, 3, Basrah I, 4, Rangoon Smooth, 5, Rangoon Rough (1), 6, Rangoon Rough (2), 7 (a, b, c), 1617, 8 (a, b), 2027, 9 (a, b), 505, 10 (a, b, c), W 880

fractions may be correlated with two sets of observations which have repeatedly been made on the bacteria in general, namely, that their serological reactions are surface reactions, and that in these reactions the specific carbohydrates play an outstanding part If 'A' and 'B' represent the actual surface layers, the relatively large amounts of specific polysaccharide which they contain are to be expected Along the same line is the observation that the Residues, relative to

their amounts, contain vanishingly small quantities of specific substance, and as we have shown below, they are almost inert serologically

We have also considered the agglutinability of this group of vibrios in relation to the relative distribution of reducing substances in their fractions   Cross-agglutinations were carried out within the group, and the results, expressed as the maximum titres obtained after 3 hours at 56°C , followed by a final reading after 18 hours in the ice-box, are given in Table V —

TABLE V

Cross-agglutination titres of the vibrio strains

Antigens	ANTI SERA						
	1617	Rangoon Smooth	Rangoon Rough (2)	2027	W 880	El Tor	Agglutinability with anti cholera sera
1617	2,000	400	0	400	0	500	Agglutinable
Rangoon Smooth	2,000	5,000	0	500	0	0	Agglutinable
Rangoon Rough (1)	0	0	0	0	0	0	Inagglutinable
Rangoon Rough (2)	0	0	4,000	0	0	0	Inagglutinable
Basrah I	1,000	0	0	0	0	0	Irregular *
2027	1,000	1,000	0	2,000	200†	0	Agglutinable
505	2,000	1,000	0	500	0	0	Agglutinable
W 880	0	0	0	0	5,000	0	Inagglutinable
El Tor	400	500	0	500	0	4,000	Agglutinable
79 B	1,000	1,000	0	1,000	0	0	Agglutinable, but irregular

* Basrah I agglutinates irregularly in the sense that the agglutinability, which is usually negative, may in an occasional sub culture be positive

† This cross reaction is of interest in view of the fact that these two vibrios, one from water and the other from cholera, have the same type of arabinose containing specific substance

It should be noted that the reactions recorded in Table V were done with young living cultures   If the antigens are old or have been killed by heat the agglutination titre of many vibrios is sometimes greatly reduced or may even be absent   This observation holds especially for strain 79-B and is also true to a lesser degree for 505 , the latter when heated at 60°C for one-half hour becomes inagglutinable with Rangoon Smooth anti-serum, whereas in the living condition it agglutinates as the

Table shows at 1   1,000   It is possible that this phenomenon may give a further lead in the question of the relationship between vibrio structure and agglutinability, and a study of it is now being made

Although the agglutination titres show the variability and specificity which is consistently found among the vibrios, it is possible to designate the following strains as agglutinable   1617, Rangoon Smooth, 2027, 505, 79-B and El Tor, the agglutinogenic power of the latter is low   The other strains show irregular agglutination (Basrah I) or absence of this property [Rangoon Rough (1) Rangoon Rough (2), and W 880], except to their own anti-sera   When the vibrios are grouped on this basis in conjunction with the relative preponderance of reducing substance in 'A' or 'B' the results are as given in Table VI —

TABLE VI

Relationship between relative distribution of reducing substance and agglutinability in the vibrios

Strain	Agglutinability	Difference between the relative amounts of reducing substances in 'A' and 'B' fractions ('A'—'B')	Reaction to Millon's reagent	Vibrio group number
1617	Agglutinable	+ 50	Smooth	I
1617	,,	+103	,,	I
1617	,,	+ 40	,,	I
Rangoon Smooth	,,	+ 11	,,	I
2027	,,	+ 34	,,	II
2027	,,	+144	,,	II
505	,,	−63	Smooth Rough	II
505	,,	−45	,, ,,	II
El Tor	,	−421	Rough	IV
79-B	,,	−708	Smooth Rough	IV
Rangoon Rough (1)	Inagglutinable	−145	Rough	I
Rangoon Rough (2)	,,	−223	,,	V
Basrah I	Irregular	−480	Smooth Rough	I
W 880	Inagglutinable	−729	,, ,,	III
W 880	,,	−100	,, ,,	III
W 880	,,	−183	,, ,,	III

Three of the agglutinable strains (1617, Rangoon Smooth and 2027) show relatively more reducing substance in ' A ' than in ' B ' fraction, while the reverse is the case in the other three strains in this category. All of the inagglutinable strains have less carbohydrate in ' A ' than in ' B '. All the organisms which are smooth in the reaction with Millon's reagent contain more reducing substance in ' A ' fraction than in ' B ' fraction, while in those giving the smooth-rough and rough reactions the reverse is the case. This finding is perhaps a further indication that ' A ' fraction and its reducing substance is predominantly at the surface of the vibrios, since the reactions with Millon's reagent, like the serum reactions, are probably surface phenomena.

The possible significance of the data in Table VI cannot be evaluated until more is known about the position of the ' A ' and ' B ' fractions in the vibrios, and their relation to agglutination ; it is probable that this knowledge will come from the study of strains like El Tor and 79-B.

SEROLOGICAL REACTIONS OF THE WHOLE VIBRIOS AND FRACTIONS

Rabbits were immunized by the intravenous injection of the whole vibrios (1617 and W 880) and by the ' A ', ' B ' and Residue fractions of these. The doses of the fractions were 0 25 c c , 0 4 c c and 0 5 c c at five-day intervals, followed a week later by doses of 0 5 c c and then 0 75 c c at weekly intervals for the four succeeding weeks. The animals were bled a week later. When 0 5 c c was given as a first dose all of the rabbits became ill and those receiving 1617 ' B ' and W 880 ' B ' died within a few hours. The two strains 1617 and W 880 were chosen as the sources of anti-sera to themselves and their fractions because they appeared to be good examples of the contrasting types of cholera and water-vibrios which we are studying. They are chemically quite distinct, 1617 having Protein I and W 880 Protein II, and the former the galactose- and the latter the arabinose-containing carbohydrate ; and finally they have shown very little variation and no irregularity in their characteristics since isolation.

The reactions used were those of agglutination where whole vibrios were concerned and of precipitation where the fractions were being studied. The precipitin reaction was carried out by adding equal quantities of the undiluted sera and the clear solution of the extracts in water together, and incubating at 56°C for 3 to 5 hours. The final readings were made after the tubes had stood overnight in the refrigerator. As Table VII shows, two types of precipitate appeared, floccular and granular. The floccular type was usually present after the tubes had been one hour in the water-bath, and the granular type appeared only after three hours and was better developed if a five-hour period was used. The controls were negative in every case.

Several points from Table VII may be briefly noted :—

Anti-sera.—1. W 880 whole anti-serum reacts only with its own organism and with none of the other whole organisms. Anti-serum to W 880 ' A ' fraction reacts with all the whole organisms in the group. The same is true of 1617 ' A ' anti-serum which reacts with W 880 whole and Rangoon Rough (2) whole, while the anti-serum to 1617 whole does not react with either of these organisms.

TABLE VII.

Agglutination and precipitin reactions between the anti-sera to whole vibrios and fractions of 1617 and W 880, and fractions from a series of vibrios

Antigens	ANTI SERA TO							
	1617 Whole	W 880 Whole	1617 'A'	W 880 'A'	1617 'B'	W 880 'B'	1617 Residue	W 880 Residue
1617								
Whole	++++ F	0	+++ F	+++ F	+++ F	0	+++ F	0
'A'	0	0	+++ F	+ G	0	+++ F	+++ F	0
'B'	0	0	0	0	0	0	0	0
Residue	0	0	0	0	0	0	0	0
W 880								
Whole	0	++++ F	+++ G	++++ F	+++ F	+++ F	++ F	+++ F
'A'	0	+++ F	+++ F	+++ F	++ F	++ F	0	+++ F
'B'	0	0	0	0	+ G	++ G	0	+ G
Residue	0	0	0	0	0	0	0	0

	Rangoon Smooth				Rangoon Rough (2)				El Tor			
	Whole	'A'	'B'	Residue	Whole	'A'	'B'	Residue	Whole	'A'	'B'	Residue
1	++ F	0	0	0	+++ G	0	+ G	0	++ G	+ G	0	0
2	++ G	0	0	0	++ G	0	0	+ G	+++ G	+ G	0	0
3	++ F	++ F	0	0	0	0	0	0	+ G	0	0	0
4	++ G	0	+ G	0	0	0	0	+ G	++ G	+ G	0	0
5	++ G	0	0	0	++ G	+ G	0	0	++++ G	+ G	0	0
6	++ F	++ G	0	0	+++ G	+ G	0	++ G	++++ G	++ G	0	0
7	0	0	0	0	0	0	0	0	0	+ G	+ G	0
8	+++ F	0	0	0	0	0	0	0	++ F	0	0	0

2   1617 'B' anti-serum reacts with the whole organisms throughout the group with the exception of Rangoon Rough (2), and the W 880 'B' anti-serum does not react with this organism or with 1617 whole   The 'B' anti-sera are somewhat less reactive than the 'A' anti-sera

3   The anti-sera to the residues of 1617 and W 880 react to about the same extent as those of the 'B' fractions   The reason for the irregularities of the cross-reactions of the four anti-sera to the 'B' and Residue fractions is not evident from the data

Antigens —1   The whole organisms are the most reactive antigens, the only failures in this respect, outside of those where anti-sera to other whole organisms were used, being with anti-sera to the two 'B' fractions [W 880 'B' vs 1617 whole , 1617 'B' and 880 'B' vs Rangoon Rough (2)]

2   The 'A' fractions are almost as reactive as the whole organisms, especially with 'A' anti-sera   With the 'B' and Residue anti-sera the reactions of these fractions are irregular

3   The 'B' fractions are only about one-third as reactive as the 'A' fractions, that of 1617 not reacting in any of the tests, while where reactions do occur they are of the slow-appearing, granular type

4   The Residue fraction is nearly inert as antigen in these reactions, it is negative in all cases except that of Rangoon Rough (2)

In general it may be said that as antigen in these reactions none of the fractions is as good as the whole vibrios   Next to it, and giving 20 reactions against the 30 shown by the whole organisms, is the 'A' fraction   The 'B' and Residue fractions have six and three reactions respectively and are thus almost inert , at the same time they are capable of giving rise to active anti-sera

The anti-sera to the fractions are more reactive, because less specific, than those to the whole vibrios

With reference to the kind of precipitate formed, there is again no body of facts on which to base an explanation of the observed distribution of the floccular and granular types   In general the whole and 'A' antigens of 1617 and W 880 give floccular reactions, the Rangoon Smooth antigens are intermediate, and the rough strains, Rangoon Rough (2) and El Tor, are largely granular as far as these two antigens are concerned , but this distinction is not clear cut   On the other hand, the few reactions in which the 'B' and Residue antigens of all the organisms are concerned are of the granular type   The existence of a relationship between these observations and the floccular and granular types of agglutination on the one hand and the distribution of the 'A' and 'B' fractions and their specific carbohydrates on the other appears to be a possibility, and is now being investigated

DISCUSSION

The question has naturally arisen with regard to the reducing substances themselves as to whether, in the case of the 'A' and 'B' fractions, these actually represent the specific carbohydrates or whether some of the reducing power observed may be the result of hydrolysis of the carbohydrate portion of the nucleo-proteins   The type of hydrolysis which we have used is much less drastic than that necessary to hydrolyse proteins, which usually require at least 6 hours on the sand-bath with

20 per cent acid, while in our work the hydrolysis has been carried on with 3 per cent acid in the water-bath for two and one-half hours   However, in order to see whether the protein was yielding reducing substance under the conditions of the experiments, in two cases the specific carbohydrate was not extracted from the Residue protein, but the whole fraction was itself hydrolysed in the usual way and the reducing power then determined   The results, which concern the second of the three determinations of 1617 and W 880 in Table III, show that hydrolysis of whole protein instead of increasing the amount of reducing substance found appear to make it somewhat less, in comparison with the experiments in which the carbohydrate itself was hydrolysed   On the basis of these findings we may conclude that an invalidation of our results through the hydrolysis of protein is improbable

SUMMARY

The acid-soluble ' A ' and ' B ' portions of a series of vibrios have been studied with respect to their content of reducing substance (carbohydrate) and a comparison made between these amounts and the amount of substance left in the Residue after the extraction of the two fractions   It is found that reducing substance is present in both the fractions and in the Residue and that the quantity in the latter is minute in proportion to the amount of the latter, which averages 85 per cent of the whole vibrio   The ' A ' and ' B ' fractions have on the other hand proportionately large amounts of reducing substance   There is some indirect evidence that these two fractions, especially that designated ' A ', represent the outer parts of the vibrio The ' A ' fraction approaches the whole vibrio in its serological activity, while, as antigens in test-tube reactions, the ' B ' and Residue portions are almost inactive, although capable of giving rise to active, non-specific anti-sera   The anti-sera to the ' A ' fraction is also non-specific

With respect to the distribution of reducing power in ' A ' and ' B ' there appears to be a parallelism between smoothness as shown by the reaction to Millon's reagent and the presence of more reducing substance in ' A ' than in ' B ' fraction   Where the amount of reducing substance is greater in ' B ' than in ' A ' the organisms are smooth-rough or rough   There is not, however, a complete parallelism between agglutinability and the possession by ' A ' of the larger amounts of reducing substance   The non-agglutinating strains in our series have less reducing substance in ' A ' than in ' B ', but of the six agglutinating strains, three have more in ' A ' and three less

Evidence is given to show that the source of the reducing power is the hydro-lysed specific polysaccharide of the vibrio

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STUDIES ON THE ANTIGENIC STRUCTURE OF *VIBRIO CHOLERÆ*

Part IX.

DISSOCIATION AND CHANGES IN CHEMICAL STRUCTURE

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WE are presenting in this paper the results of our investigations on the proteins and carbohydrates of sixteen vibrios, which we have studied along lines similar to those laid down in a previous communication (Linton, Mitra and Shrivastava, 1934*b*) The organisms reported on here have been chosen chiefly on the basis of their variability, as in this way we have been able to note some of the chemical changes which underlie dissociation We have also included in the ' Discussion ' a brief summary of the results of the work and have made an attempt to correlate these with the serological reactions The vibrio strains used include four Rangoon strains, four Basrah strains, four El Tor strains, and a group of four other strains which present points of interest

THE RANGOON STRAINS

Rangoon Smooth and Rangoon Rough [designated Rangoon Rough (1) in this paper] were both isolated from a plate which had been streaked with stool material from a non-fatal case of cholera imported into Rangoon from Calcutta , the former was agglutinable, the latter non-agglutinable We received these two strains from the Central Research Institute, Kasauli, in September 1933 These vibrios

underwent the usual routine sub-cultures and on a plate streaked with Rangoon Rough (1) in January 1934, a 'medusa-head' colony was found, and the strain of which this colony was the origin was designated Rangoon Rough (2)   Later on, Rangoon Rough (2) was itself intensively sub-cultured, as described below, and in June 1934 and again in October 1934 a smooth agglutinating strain was isolated from it   This was designated Rangoon Rough (2*a*)   These four strains constitute the Rangoon group, and they form an interesting series for the study of the chemical basis of variation

The characteristics of these four strains are compared in Table I

Rangoon Smooth (Plate XXIII, fig 1) is in many ways a typical example of the vibrios isolated in the majority of cases of cholera   It has the usual colony morphology and the appearance in stained smears associated with *Vibrio choleræ*, very active motility and a single flagellum   It is salt-stable, smooth when tested with Millon's reagent, gives the typical sugar reactions, indol production and type of growth in peptone water   It is both proteolytic and slightly hæmolytic, the latter power having been gained since its first isolation   It is resistant to bacteriophage types A, H and K (Shillong nomenclature)

Rangoon Rough (1) (Plate XXIII, fig 2) is a strain having many similarities to the usual run of rough dissociants   In morphology it shows considerable pleomorphism , most of the vibrios are without flagella and the culture is accordingly almost non-motile   Its sugar reactions, growth in peptone water and indol production are like those of the smooth homologue, but it is unstable in saline and rough to Millon's reagent   The hæmolytic power is much stronger than that of Rangoon Smooth, while the proteolytic power is the same   It is non-agglutinating with anti-serum to Rangoon Smooth and with anti-sera to a number of other vibrios having the smooth characteristics   It is interesting to note that in the shift toward roughness the strain has become resistant to eight of the ten types of cholera bacteriophage, in contrast to the resistance of Rangoon Smooth to three types

Rangoon Rough (2)   This strain shows a greater deviation from the smooth type than any other with which we have worked   The colonies are of the dry, corrugated, 'medusa-head' type (Plate XXIII, fig 3), resembling the Variant I of Balteanu (1926), and also a form described by Eisenberg (1912)   It is more motile than its immediate parent Rangoon Rough (1), but less so than the smooth homologue Rangoon Smooth   Biochemically it does not produce indol at any period up to 10 days, nor does it ferment any sugar except glucose, and that too only after 72 hours' incubation   It grows slowly in peptone water with the formation of a thick dry pellicle, and with only a slight turbidity in the rest of the tube   It has no proteolytic power on serum or gelatin, nor does it hæmolyse sheep's blood agar, except around certain areas where dissociation has occurred   In contrast to the other strains it is resistant to all ten types of bacteriophage   The tendency toward further dissociation is strongly marked in this strain   During the first six months after isolation, dissociation could be observed in the appearance, around some of the rough colonies after they had incubated for several days, of a moist semi-transparent growth, and from this fringe the first strain of Rangoon Rough (2*a*) was isolated   It was these fringes which were hæmolytic   The serological reactions of this strain are described below.

PLATE XXIII
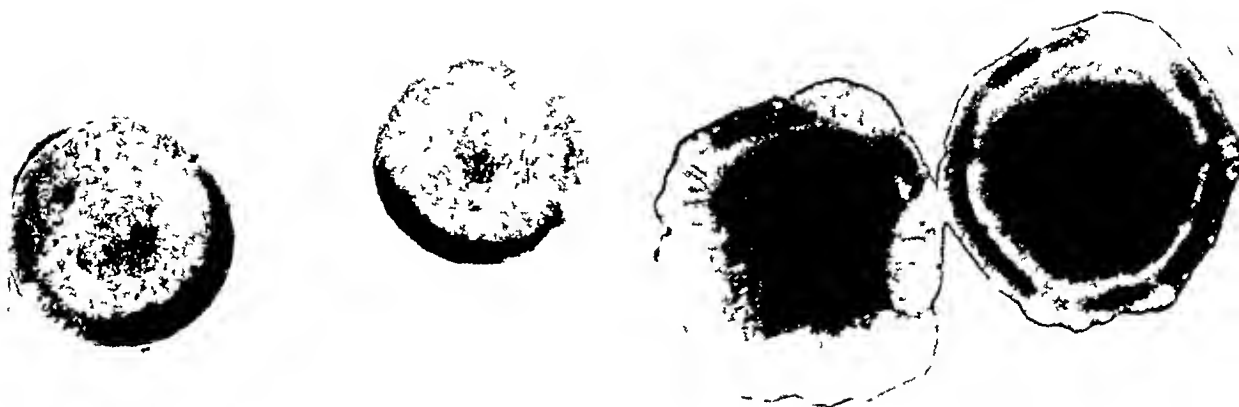
Fig 1   Colonies of Rangoon Smooth on agar

Fig 2   Colonies of Rangoon Rough (1) on agar

Fig 3   Colonies of Rangoon Rough (2) on agar

TABLE I

Characteristics of the Rangoon strains

	Rangoon Smooth	Rangoon Rough (1)	Rangoon Rough (2)	Rangoon Rough (2a)
Colonies on agar	Smooth	Rough	Dry, rugose, 'medusa head' type	Rough opaque white colony with a smooth element
Morphology	Typical Gram negative vibrios	Predominantly like the smooth strain, but showing considerable pleomorphism. Gram negative	Thick, small, slightly curved rod. Gram negative	Pleomorphic, Gram-negative
Flagella	Single terminal flagellum	Mostly non flagellated	Single terminal flagellum	Single terminal flagellum
Motility	Actively motile	Very few motile forms	Moderate motility	Actively motile
Peptone water, 24 hours	Uniform turbidity with pellicle formation	Uniform turbidity with pellicle formation	Heavy dry pellicle and thin turbidity in the remainder of the medium	Thin turbidity with formation of a thin pellicle
Indol	Positive	Positive	Negative	Negative
Millon's reagent	Smooth	Rough	Rough	Rough
Sugar fermentations	Typical	Typical	Glucose only, after 72 hours	Glucose and mannite only, after 72 hours
Proteolysis— serum	Positive	Positive	Negative	Negative
gelatin	Positive	Positive	Negative	Negative
Haemolytic power (sheep's blood agar)	Positive (slight)	Positive (very vigorous)	Negative, except around areas where dissociation is occurring	Positive
Bacteriophage	Resistant to 'phages A, H and K	Resistant to 'phages A, B, C, D, E, G, H and K	Resistant to all ten types of 'phage	Resistant to all ten types of 'phage
Salt stability	Stable	Unstable	Unstable	Unstable

During the last few months, dissociation in Rangoon Rough (2) has apparently become more widespread, as now on any plate streaked with it only about 30 per cent of the colonies are of the 'medusa-head' type, the remainder being more like the Rangoon Rough (1) colonies in appearance

The slowness of growth of Rangoon Rough (2) is one of its outstanding characteristics   An ordinary mass planting, using about 150 bottles of Rangoon Smooth or Rangoon Rough (1) yields about 24 g of bacteria (dry weight)   Under the same conditions, except that growth is allowed to continue for four days instead of two, Rangoon Rough (2) yields only about 4 g of dried bacteria   With such a difference in speed of growth it is conceivable that Rangoon Rough (2) might lie hidden in a culture of Rangoon Smooth or Rangoon Rough (1) and that only accidentally and infrequently would streaking of one of these cultures succeed in isolating it as a distinct colony   Rangoon Rough (2) does not agglutinate with anti-sera to Rangoon Smooth or Rangoon Rough (1), and with its own anti-serum it reacts slowly to form the granular type of agglutination

Rangoon Rough (2a)   This vibrio, whose origin has been described above, was isolated in an attempt to obtain a strain which had reverted, at least in part, toward the smooth condition   The colonies on agar are smooth-rough, like those of Rangoon Rough (1), although more opaque   They appear to resemble the Variant III colonies of Balteanu (*loc cit*)   The organism itself is an actively motile curved rod, somewhat larger than the usual vibrio   In peptone water it grows thinly but diffusely and forms a slight pellicle   It is rough to Millon's reagent, does not produce indol, but has, in contrast to its parent, the power of fermenting mannite   It is negative in the proteolytic tests but has regained the power of hæmolysis   Like its immediate parent it is resistant to all the ten types of cholera bacteriophage

We may conclude that Rangoon Rough (2a) has shown the desired reversion toward smoothness in the following respects   active motility, diffuse growth, increased power of fermentation and positive reaction in the hæmolytic test   On the other hand it resembles its immediate parent in failure to produce indol, roughness to Millon's reagent, negative proteolysis and complete resistance to the bacteriophage types

In general, the Rangoon strains represent a series which runs from smoothness to extreme roughness and from the latter partially reverts toward the smooth type again   We have studied the proteins of the first three members of the group, and the carbohydrates and serological relationships of all of them

*Proteins —*We have already detailed the methods, based on the work of Woodman (1921), which we have used for the differentiation of the vibrio proteins (Linton Mitra and Shrivastava, 1934b)   In brief, these consist of the collection of about 40 g (dry weight) of the growth of each vibrio   The globulin is separated out by repeated precipitation with ammonium sulphate, dialysed against running water, followed by distilled water, until sulphates can no longer be detected, and the pseudoglobulin and euglobulin fractions separated, washed and dried   In none of the vibrios did more than a trace of albumin appear to be present, and in most of them it was entirely absent

The globulins were taken up in $N/2$ NaOH to form a 1 per cent solution, and polarimetric readings taken at intervals over a period of about 300 hours   The

protein solutions are kept at 37°C during this period   It was found that the optical activity of the solutions decreased, rapidly in the first twenty-four hours and more slowly thereafter, until after about 220 hours no further change occurred   The rate of change in optical activity plotted against the time always yielded a perfectly smooth curve, and we have found that only two kinds of curve are obtained   The protein giving the first of these we designated Protein I and that giving the second curve, Protein II   Protein I is the protein generally found in the cholera vibrios and Protein II in the water-vibrios and the El Tor vibrios   It is possible that the two types of protein are the result of the different manner in which the same kinds of amino-acids are grouped together within the molecule (Linton, Mitra and Shrivastava, 1934a)   The rate of change in optical activity is a function of the rate of change within the protein molecule under the influence of alkali and will thus vary with the structure of the molecule (Woodman, 1921 , Jordan-Lloyd, 1926)   The method is particularly useful in studying the relationships of aberrant organisms and dissociants, as it yields data which cannot be obtained by the serological reactions

The pseudo- and euglobulins of Rangoon Smooth, Rangoon Rough (1) and Rangoon Rough (2) were prepared as described above and the polarimetric readings during racemization in dilute alkali were obtained   These data are given in Table II —

TABLE II

Specific rotations of the globulin fractions of three Rangoon strains in N/2 alkali

Hours	RANGOON SMOOTH		RANGOON ROUGH (1)		RANGOON ROUGH (2)	
	Pseudo globulin,	Euglobulin	Pseudo globulin	Euglobulin	Pseudo globulin	Euglobulin
5			−64°	−64°	−60°	−60°
24	−42°	−43°	−43°	−43°	−39°	−38°
48	−35°	−35°	−35°	−35°	−31°	−31°
72	−29°	−30°	−29°	−30°	−25°	−25°
96	−26°	−26°	−26°	−26°	−22°	−22°
120	−25°	−25°	−25°	−25°	−20°	−20°
144	−23°	−23°	−23°	−23°	−18°	−18°
172	−21°	−21°	−21°	−21°	−17°	−17°
196	−20°	−20°	−20°	−19°	−16°	−16°
265	−19°	−19°	−19°	−19°	−14°	−14°

After the period of 265 hours the readings were constant in each case

As the above table indicates, the racemization data for the proteins of Rangoon Smooth and Rangoon Rough (1) are identical and accordingly it is probable that the two have the same molecular structure A comparison of these data with those obtained in our previous work will show that the proteins of the two are identical with that which we have designated as Protein I A single curve will satisfy the data given in the table for these two strains and for the Protein I-containing vibrios studied previously (Linton, Mitra and Shrivastava, 1934*b*)

On the other hand, the readings given by the globulins of Rangoon Rough (2) yield a curve which begins, proceeds and ends differently, and which accordingly must be the result of a different molecular composition in the protein Furthermore, the curve yielded by the Rangoon Rough (2) data is identical with that which we have previously found to be given by organisms possessing Protein II This observation is of interest because the water-vibrios which we have so far studied contain this same protein We cannot yet know whether the Protein II of Rangoon Rough (2) has arisen as a mutation from the Protein I of the parent strain or whether the vibrios having this protein lie hidden because of their slower growth in the cultures of the parent strain It is evident, however, that one type of protein, characteristic of the cholera vibrios, can give rise, either through mutation or because of a differential rate of growth, to a second type of protein, which is characteristic of the water-vibrios

We have again found, as in our previous work, that no differences occur in the optical activity of the pseudo- and euglobulins of any given strain

Specific carbohydrates —The specific carbohydrates of the four Rangoon strains were prepared and purified according to the methods previously described (Linton and Shrivastava, 1933*b* and 1933*c*) The hydrolysis of the substances and the identification of their constituents were also carried out in the usual way The results are given in Table III —

<div align="center">TABLE III</div>

<div align="center">*Results of the hydrolysis of the specific carbohydrates of the Rangoon strains*</div>

Strains	Weight of carbohydrate, g	Period of hydrolysis	Hydrolysis, per cent	Melting point of phenylosazone, °C	Characteristic sugar
Rangoon Smooth	1 22	10 mins	6 2	180	Galactose
Rangoon Rough (1)	0 33	10 ,,	8 5	186	,,
Rangoon Rough (2)	0 38	2 hrs	33 5	208*	Glucose
Rangoon Rough (2*a*)	1 29	2 ,,	22 6	208*	,,
Rangoon Rough (2*a*1)	0 37	2 ,,	15 3	208*	,,

* No change in melting-point when mixed with known glucose osazone.

Rangoon Smooth and Rangoon Rough (1) have the same specific carbohydrate, and this bears out our previous finding that the rough strains of the type usually found do not differ qualitatively in this respect from their smooth homologues. Where the difference appears to lie is in the amounts of specific substance present in the two. In the case of the Rangoon strains, as Table IV shows, 25 g. of Rangoon Smooth yielded 1·22 g. of specific substance (4·8 per cent), while 39 g. of Rangoon Rough (1) gave only 0·33 g. (0·8 per cent), about one-sixth as much in respect to the proportionate weights. Since, as we have shown above, the proteins of the two organisms are the same and their polysaccharides are also qualitatively the same, it is probable that at least one factor in the non-agglutinability of Rangoon Rough (1) by Rangoon Smooth anti-serum is the smaller amount of specific substance in the rougher strain. It is possible that another factor which has to be considered is the difference in the distribution of the specific carbohydrate in the two organisms. A beginning in the study of this factor has already been made (Linton and Mitra, 1934; Linton, Mitra and Seal, 1935).

The finding in Rangoon Rough (2) and Rangoon Rough (2a) of a sugar, whose phenylosazone had the characteristic melting-point of glucose, was of considerable interest, and a more detailed study was carried out. Much time was consumed in obtaining sufficient carbohydrate because of the scantiness and slowness of the growth of Rangoon Rough (2), but eventually 8·0 g. of purified polysaccharide were collected and the analysis of this material was carried out in duplicate, about 4·0 g. being used each time. These analyses gave concordant results.

4·222 g. of the polysaccharide were taken up in 10 c.c. of 50 per cent H_2SO_4, and kept at 37°C for 18 hours. At the end of this time the reducing power was 10·9 per cent (460 mg.) calculated as glucose. After dilution to bring the acid concentration to 3 per cent, the mixture was heated on the water-bath for two hours: reducing power, 12·2 per cent (514 mg.); after a further heating for two hours the reducing power was 9·4 per cent (415 mg.) and the hydrolysis was stopped.

The hydrolysate was neutralized with $CaCO_3$, the calcium sulphate filtered off and washed free from reducing substances. The filtrate after being decolorized with charcoal was evaporated to a small volume at low temperature under vacuum. Four volumes of absolute alcohol were then added to it, but the expected precipitate of calcium aldobionate was not obtained. The solution presumably containing the aldobionic acid had a reducing value equivalent to 7·6 mg. of glucose. After two hours' hydrolysis this increased to 9·0 mg. and then decreased to 6·0 mg. after four hours, when the experiment was terminated. Again in the second attempt to obtain the calcium aldobionate, in which 4·0 g. of the specific carbohydrate was used, no precipitate of the salt could be obtained. In this trial the solution of ' aldobionic acid ' had no reducing power to begin with, the equivalent of 9·0 mg. after two hours' hydrolysis, and was again negative after five hours' heating. The results of these attempts to isolate and hydrolyse the aldobionic acid constituent may be compared with the cases in which this component was obtained (Linton and Shrivastava, 1933a). In these cases the aldobionic acid was readily separated as the calcium salt, and after decomposition it hydrolysed slowly over a period of 19 to 21 hours and increased between 100 per cent and 300 per cent in reducing power. It is probable that the low reducing values obtained from the presumed aldobionic acid of the

Rangoon Rough (2) carbohydrate represent only reducing substances which had been carried over from the hydrolysate

The portion of the hydrolysate remaining after the attempted separation of the calcium aldobionate was then studied, and as it had a high content of reducing substance (415 mg) it appeared that the most suitable way to identify its contents was to obtain them in crystalline form   The solution itself yielded a phenylosazone melting sharply at 210°C   The solution was concentrated *in vacuo* to a syrupy consistency, seeded with a few crystals of glucose, a little absolute alcohol added, and the mixture placed in the refrigerator under vacuum   After four days a solid white crystalline mass was present   The crystals were washed with a little ethyl alcohol, followed by methyl alcohol and finally with ethyl alcohol and dried *in vacuo* Yield   0 3350 g (81 per cent)

0 15 g made up to form a 1 per cent solution in distilled water gave a specific rotation of $+$ 54 0°, using a 1 dm tube and sodium light   Known glucose $+$ 52 5°

The crystals, like those of glucose, did not have a definite melting-point, but showed softening and darkening between 110°C and 125°C (Zemplén, 1922)   The phenylosazone of the crystals melted sharply at 214°C and the melting-point was unchanged when it was mixed with known glucose-phenylosazone   The characteristic crystals of potassium acid saccharate were also readily obtained

From the evidence of the direction and degree of specific rotation, the melting-points of the phenylosazones, the behaviour of the crystals themselves when heated and the formation of potassium acid saccharate, it is probable that the crystalline substance obtained from the specific carbohydrate of Rangoon Rough (2) is glucose, and further that there are no indications of other substances in this polysaccharide

What appears to have been the same type of specific carbohydrate as is present in Rangoon Rough (2) and Rangoon Rough (2a) was reported by Jermoljewa and Bujanowskaja (1930)   From a laboratory strain of *Vibrio choleræ* they isolated a carbohydrate substance which on hydrolysis yielded a sugar whose phenylosazone melted at 204°C and which itself had a specific rotation of $+$ 64 0°   These authors tentatively identified the sugar as glucose

As we have shown in Table III, the Rangoon Rough (2a) strain also possesses a glucose-containing polysaccharide   The amount of polysaccharide in Rangoon Smooth and Rangoon Rough (1) have been discussed above   Also included in Table IV are the same data on the other strains   These show that Rangoon Rough (2) in spite of its roughness yields the same percentage of specific carbohydrate as the smooth homologue—4 8 per cent   In two other trials it yielded 4 6 per cent and 4 8 per cent   This finding demonstrates again that the transition from Rangoon Rough (1) to Rangoon Rough (2) is of an entirely different type than that from Rangoon Smooth to Rangoon Rough (1)   The latter is accompanied by loss of carbohydrate and the former by the appearance of a different type of carbohydrate, but in the same quantity as in the smooth strain   The basis for variation in the vibrios is thus not single, but has at least two factors and as we shall show in considering the Basrah strains, probably three   Strain Rangoon Rough (2a) also

possesses an amount of carbohydrate (5 9 per cent) which is similar to that of the smooth strains, although as Table I shows it is not smooth in its characteristics It is possible that the significance of these amounts of carbohydrate is to be found in the work referred to above on the distribution of the carbohydrate in the vibrios

TABLE IV

Relationship between type of growth and yield of specific carbohydrate in the Rangoon strains

Strains	Type of growth on agar	Dry weight of vibrios, g	Weight of specific carbohydrate, g	Percentage of specific carbohydrate
Rangoon Smooth	Smooth	25 0	1 22	4 8
Rangoon Rough (1)	Rough	39 0	0 33	0 8
Rangoon Rough (2)	' Medusa head '	5 2	0 25	4 8
Rangoon Rough (2*a*)	Rough	6 6	0 40	5 9

The rough to smooth transition in the Rangoon strains —When first isolated as described above in June 1934 from the fringe of a 'medusa-head' colony of Rangoon Rough (2), Rangoon Rough (2*a*) agglutinated with Rangoon Smooth anti-serum at a dilution of 1 800, but the agglutination was indefinite and difficult to be sure of After two weeks of repeated sub-cultures on agar it had become definitely agglutinable at 1 6,400 with this anti-serum and was also highly agglutinable with the Rangoon Rough (2) anti-serum During the two succeeding months, however, the agglutination titre with the smooth anti-serum gradually declined until, in September 1934, it gave only an indefinite reaction at 1 100, although still highly agglutinable with the rough anti-serum

As we wished to test this strain in cross-absorption experiments, it was essential to attempt a restoration of its agglutinability For this purpose we adopted the technique of 24-hour sub-cultures in 0 5 per cent glucose broth which had been used successfully in bringing about the rough to smooth transition by Koser and Styran (1930) At intervals of five days plates were streaked from the glucose broth cultures and the colonies tested for agglutinability with the smooth anti-serum We began with three strains Rangoon Rough (1), Rangoon Rough (2*a*), which as we have just stated had been agglutinable but had become inagglutinable, and Rangoon Rough (2) From the latter, two types of colony were sub-cultured, the 'medusa-head', and the somewhat less rough type which, as already described, now makes up about 70 per cent of the colonies of this strain

All four strains were inagglutinable with Rangoon Smooth anti-serum   The results of the experiments are shown in Table V —

TABLE V

Changes induced in the agglutination reaction and in colony morphology
of rough vibrio strains by repeated sub-cultures
in 0 5 per cent glucose broth

Time	Rangoon Rough (2)		Rangoon Rough (2a)	Rangoon Rough (1)
1st day	Non agglutinable, 30 per cent 'medusa-head'	Non agglutinable, 70 per cent somewhat less rough	Non agglutinable, 100 per cent slightly rough	Non - agglutinable, 100 per cent slightly rough
5th day	Non agglutinable, 20 per cent 'medusa-head', 80 per cent less rough	Non agglutinable, 100 per cent smooth-rough	Slow, granular, agglutination at 1 800, 100 per cent bluish opalescent colonies	80 per cent smooth with a slight rough element, agglutinable at 1 100, 20 per cent as before, non agglutinable
10th day	No 'medusa-head' type, 20 per cent rough, 80 per cent smooth, all non-agglutinable	Agglutinable at 1 100, smooth rough	Agglutinable at 1 2,000, 100 per cent smooth-rough	Agglutinable at 1 200, poor growth
15th day	10 per cent rough opaque colonies, non agglutinable, 90 per cent smoother than before, agglutinable at 1 200	95 per cent bluish colonies, agglutinable at 1 500, 5 per cent 'medusa-head' colonies, non-agglutinable	No change, agglutinable at 1 2,000	Died out
20th day	100 per cent smooth colonies with a slight rough element, agglutinable at more than 1 400	90 per cent smooth colonies, agglutinable at 1 500, and 10 per cent 'medusa-head' colonies, non agglutinable	75 per cent smooth and agglutinable at more than 1 2,000, 25 per cent smooth rough and less agglutinable	

As Table V shows, it was possible by repeated sub-cultures during 20 days to turn an inagglutinable into an agglutinable strain in every case, and also to modify the colony morphology in the direction of greater smoothness The strain attaining the highest titre with the smooth anti-serum—Rangoon Rough (2a)— was used in the serological experiments detailed below Some other points are of

interest in the table   The strain derived from the smoother type of colony in Rangoon Rough (2) gave after 15 days a number of ' medusa-head ' colonies again, and at 20 days their number was doubled   It appeared that the equilibrium between the kind of organism yielding this type of colony and that giving the smoother type was beginning to shift again toward the rough side   In the ' Discussion ' in this paper we have included this shift in type of growth within a strain as one of the means of variation in the vibrios   The poorest response to the sub-culturing was given by the Rangoon Rough (1) strain, which attained an agglutination titre of only 1   200, although its colonies became smoother in appearance   It grew very scantily in these sub-cultures and died out completely between the 10th and 15th days   It is possible that this susceptibility to the environment is the result of its low content of specific substance

Work along the lines of these experiments is being continued

Serological reactions in the Rangoon group

Anti-sera were obtained in rabbits against Rangoon Smooth, Rangoon Rough (1) and Rangoon Rough (2), and these were used in direct agglutinations and in cross-absorption experiments as shown in Table VI —

Table VI

Direct agglutinations and cross-absorptions in the Rangoon group

A   Direct agglutinations

	Strains			
	Rangoon Smooth	Rangoon Rough (1)	Rangoon Rough (2)	Rangoon Rough (2*a*)
Anti sera to				
Rangoon Smooth	6,400	0	0	3,200
Rangoon Rough (1)	200	3,200	0	0
Rangoon Rough (2)	0	0	1,600	1,600

Rangoon Smooth anti-serum agglutinates its own vibrio and Rangoon Rough (2*a*)

Rangoon Rough (1) anti-serum agglutinates its own vibrio, and shows a low titre reaction of the granular type with Rangoon Smooth   This cross-reaction is a development during the months from June to October 1934

Rangoon Rough (2) anti-serum agglutinates its own organism and Rangoon Rough (2*a*)   It is thus evident that Rangoon Rough (2*a*) reacts with anti-sera against two strains which will not themselves cross-react

B Absorption of Rangoon Smooth anti-serum

	STRAINS			
	Rangoon Smooth	Rangoon Rough (1)	Rangoon Rough (2)	Rangoon Rough (2a)
Rangoon Smooth anti serum absorbed with				
Rangoon Smooth	0	0	0	0
Rangoon Rough (1)	1,600	0	0	0
Rangoon Rough (2)	6,400	0	0	0
Rangoon Rough (2a)	3,200	0	0	0

Rangoon Smooth anti-serum absorbed by its homologous organism completely loses the agglutinins for itself and for Rangoon Rough (2a), and its titre for itself is reduced by absorption with Rangoon Rough (1)

Rangoon Smooth anti-serum absorbed by Rangoon Rough (2) shows no reduction in its titre for itself, but its agglutinins for Rangoon Rough (2a) have been removed

Rangoon Smooth anti-serum absorbed by Rangoon Rough (2a) has a lowered titre for Rangoon Smooth itself and no agglutinins for Rangoon Rough (2a)

In short, Rangoon Smooth appears to be connected serologically with Rangoon Rough (1) and with Rangoon Rough (2a) but with Rangoon Rough (2) the connection is not direct but through Rangoon Rough (2a)

C Absorption of Rangoon Rough (1) anti-serum

	STRAINS			
	Rangoon Smooth	Rangoon Rough (1)	Rangoon Rough (2)	Rangoon Rough (2a)
Rangoon Rough (1) anti serum absorbed with				
Rangoon Smooth	0	3,200	0	0
Rangoon Rough (1)	0	0	0	0
Rangoon Rough (2)	200	3,200	0	0
Rangoon Rough (2a)	200	3,200	0	0

Rangoon Rough (1) absorbed by Rangoon Smooth loses its low content of agglutinins for the latter, but is unchanged toward itself , absorbed by itself it loses its titre against both itself and Rangoon Smooth

Rangoon Rough (1) absorbed by Rangoon Rough (2) does not have its low titre against Rangoon Smooth lessened, nor are its agglutinins for itself reduced at all   Rangoon Rough (1) absorbed with Rangoon Rough (2a) does not lose its agglutinins against Rangoon Smooth or itself

These findings appear to show that Rangoon Rough (1) is connected serologically with Rangoon Smooth, but is not connected with Rangoon Rough (2) or Rangoon Rough (2a)

D   Absorption of Rangoon Rough (2) anti-serum

	STRAINS			
	Rangoon Smooth	Rangoon Rough (1)	Rangoon Rough (2)	Rangoon Rough (2a)
Rangoon Rough (2) anti serum absorbed with				
Rangoon Smooth	0	0	1,600	800
Rangoon Rough (1)	0	0	1,600	1,600
Rangoon Rough (2)	0	0	0	0
Rangoon Rough (2a)	0	0	0	0

Rangoon Rough (2) absorbed by Rangoon Smooth shows no reduction in the titre for itself but its titre for Rangoon Rough (2a) is reduced   When Rangoon Rough (2) anti-serum is absorbed by Rangoon Rough (1) the titre is unchanged

Rangoon Rough (2) absorbed by itself completely loses its agglutinins for itself and for Rangoon Rough (2a)   The same complete loss occurs when the absorption is carried out with Rangoon Rough (2a)

It is interesting to compare these serological relationships with the chemical relationships, in so far as the latter are known   It should be stated that the variability of Rangoon Rough (2a) is such that we have been unable to attempt the collection of the large amounts necessary for its protein analysis   It is probable that in time the strain will reach stability, but until it does it is not possible to go further in the attempted correlations than we have in the succeeding Table VII, which also serves to summarize the information on the serology of the Rangoon strains given in Table VI

<div align="center">TABLE VII</div>

Correlation between the serological and chemical relationships of the Rangoon strains

		Serological relationship	Chemical relationship
Rangoon Smooth and	Rangoon R (1)	+	+
	Rangoon R (2)	0	0
	Rangoon R (2a)	+	Unknown
Rangoon Rough (1) and	Rangoon S	+	+
	Rangoon R (2)	0	0
	Rangoon R (2a)	0	Unknown
Rangoon Rough (2) and	Rangoon S	0	0
	Rangoon R (1)	0	0
	Rangoon R (2a)	+	+
Rangoon R (2a) and	Rangoon S	+	Unknown
	Rangoon R (1)	0	0
	Rangoon R (2)	+	+

With the above-noted exception of the chemical relationship of Rangoon Rough (2a) to the others, the correlation between serological reactions and chemical structure in the Rangoon strains is complete It also appears that as in the case of the biochemical and morphological characteristics, so in the serological reactions, Rangoon Rough (2a) has reverted in part from its very rough parent strain toward the smooth, agglutinable Rangoon Smooth

<div align="center">THE BASRAH STRAINS</div>

When these four strains, which we received from the Central Research Institute, Kasauli, were isolated from cholera cases at Basrah in 1931, they were all agglutinable at a 1 4,000 dilution Two months later they had become inagglutinable with any of the cholera anti-sera used, and this change was attributed by Doorenbos to the presence of cholera bacteriophage in the cultures ; details of the agglutination reactions and bacteriophage contaminations are given by Panayotatou (1931) and Doorenbos (1932) Since their isolation the strains have been studied in a number of laboratories and have proved variable both morphologically and serologically In our own work we have not found them to vary in their biochemical properties, but we have at times been unable to obtain agglutination with anti-sera to smooth cholera vibrios, a smooth water-vibrio, and an El Tor strain At other periods agglutination at a low dilution (1 200) would occur with all these sera except that against the El Tor vibrio We were unable to find anything in the conditions under which the strains were growing that would account for this variation

The characteristics of the strains are outlined in Table VIII It should be stated that such lists for strains like these can only give an approximation of some of their characteristics, especially those relating to type of growth For example,

TABLE VIII

Characteristics of the Basrah strains

	Basrah I	Basrah II	Basrah III	Basrah IV
Colonies on agar	Smooth, with a slight rough element after 48 hours	Smooth, with a slight rough element after 48 hours	Smooth	Smooth rough
Morphology	Short curved Gram negative vibrio	Pleomorphic, having both coccoid and long spirillar forms Gram negative	Pleomorphic, coccoid forms and vibrios with faintly staining centres Gram negative	Typical, Gram negative
Flagella	Single terminal flagellum	Single terminal flagellum	Flagella not found	Single terminal flagellum
Motility	Actively motile	Actively motile	Few motile organisms	Actively motile
Peptone water at 24 hours	Uniform turbidity with formation of a thick pellicle	Uniform turbidity with formation of a pellicle	Uniform turbidity with the formation of a thin pellicle	Uniform turbidity with the formation of a pellicle
Indol	Positive	Positive	Positive	Faintly positive
Millon's reagent	Smooth rough	Smooth rough	Smooth rough	Rough
Sugar fermentations	Typical in 48 hours	Typical in 48 hours	Typical in 48 hours	Typical in 24 hours
Proteolysis — serum	Positive	Positive	Positive	Positive
gelatin	Negative	Negative	Negative	Negative
Haemolytic power (sheep's blood agar)	Positive (slight)	Negative	Positive (strong)	Positive (strong)
Bacteriophage	Resistant to 'phages A, E, F, G and J	Resistant to 'phages A, E, F, G and J	Resistant to 'phages A, E, F, G and J Doubtfully resistant to type H	Resistant to 'phages A, F, F, G and J
Salt stability	Stable	Stable	Stable	Stable

the colony form of Basrah I is given as smooth, as it usually is In an occasional sub-culture, however, 75 per cent of the colonies may take the dry, rugose form found in Rangoon Rough (2), and these on a second sub-culture may revert to the smooth type again All the strains exhibit considerable morphological variation when examined in stained slides at weekly intervals All are heavily contaminated with bacteriophage (the Shillong nomenclature is followed in the table) and the number and type of bacteriophages to which they are resistant varies from time to time For example, when Basrah I was tested in February 1934, it was resistant to types A, E, F, G and J Tested again in August 1934, the strain was found to have become in addition resistant to types C and D The lack of correlation between proteolytic and hæmolytic power which was noted in the vibrios by Baujean (1913) is also exhibited by the Basrah strains Growth in peptone water, indol production and sugar fermentations are slow, and in Basrah IV the power of indol production is very poorly developed

The proteins and carbohydrates of these strains were studied by the methods used in our previous work

Proteins —The racemization data are given in Table IX For the sake of brevity the table includes only the data given by the euglobulin fractions in N/2 NaOH Exactly similar data were given by the pseudoglobulin fractions in the same concentration of alkali, and when the proteins were taken up in N/4 NaOH all the curves were again similar to each other and different from those in the N/2 alkali As in the case of the other vibrios, the Basrah proteins were found to consist of globulin, and albumin was usually absent or present only as a faint trace

TABLE IX

Specific rotations of the euglobulins of the Basrah strains in N/2 NaOH

Hours	Basrah I	Basrah II	Basrah III	Basrah IV
5	−59°*	−60°	−60°	−60°
24	−39°	−38°	−39°	−39°
48	−30°	−30°	−31°	−31°
96	−21°	−22°	−22°	−22°
120	−20°	−20°	−20°	−20°
196	−16°	−15°	−15°	−15°
216	−14°	−14°	−14°	−14°
265	−14°	−14°	−14°	−14°

* 5½-hour reading

It is evident from the Table that all the euglobulins from these Basrah proteins exhibit the same rate of decline in optical activity, and a comparison of these data

with those previously obtained shows that all of them have the protein which we have designated Protein II

Specific carbohydrates --Basrah I proved to possess the galactose-containing carbohydrate In Basrah II we again found evidence of glucose, the preliminary hydrolysis having yielded a phenylosazone whose melting-point of 208°C was not changed when it was mixed with known glucose-phenylosazone On account of this finding we have studied this polysaccharide in more detail

4 295 g of the gum from Basrah II were taken up in 5 0 c c of 50 per cent H_2SO_4 and placed at 37°C for 24 hours At the end of this time the solution was diluted to 100 c c and titrated reducing substances, 8 6 per cent (368 mg) The attempted preparation of the calcium aldobionate, carried out exactly as in the case of Rangoon Rough (2) and other strains, gave a final solution which had no reducing power After four hours' hydrolysis the reducing power was still negative and the experiment was not carried further It was evident throughout this experiment, as in the similar ones carried out with Rangoon Rough (2), that we were dealing with a polysaccharide of a different constitution to that which we had previously met with

The remaining hydrolysate had the same characteristics as that of the corresponding portion of the Rangoon Rough (2) hydrolysate, and gave evidence of the presence of glucose as the sole constituent melting-point of phenylosazone, 215°C It is evident that in this specific substance we were unable to demonstrate any aldobionic acid complex

Basrah III and Basrah IV have proved different from any of the 40 vibrio strains previously studied in that considerable difficulty has been experienced in obtaining any clear and consistent results in identifying the sugars present

When the first planting of Basrah III was treated by the usual methods for separation and hydrolysis, it yielded a small amount of a carbohydrate whose purified phenylosazone had a melting-point of 195°C , a temperature which does not correspond with the melting-point either of glucose- or galactose-phenylosazone, but is approximately what may be obtained from a mixture of these two osazones Mixed with the known osazones of glucose and of galactose, the melting-point was reduced in each case Two weeks later a second planting of this organism was made The growth on this occasion was rough and scanty and the yield of carbohydrate correspondingly small As the strain appeared to have shifted to the rough condition of growth, and to be remaining there, its carbohydrate was collected from further plantings, until 1 49 g of dried specific substance had been obtained This on hydrolysis yielded a sugar whose osazone melted sharply at 208°C , and this melting-point was not changed when the crystals were mixed with known glucose osazone The further identification of glucose by the formation of potassium acid saccharate was not attempted, since glycuronic acid also yields this compound and it was thought at that time that glycuronic acid might be a constituent of this glucose-containing polysaccharide, as it is of the arabinose- and galactose-containing polysaccharides

A smooth-rough colony was then picked from a plating of Basrah III, which when sub-cultured and grown *en masse* yielded a small amount of a carbohydrate On hydrolysis this gave a sugar whose phenylosazone melted sharply at 182°C , and had the physical appearance and solubilities of galactose osazone From a second

colony, which resembled the original Basrah III in morphology, the phenylosazone finally obtained had a melting-point of 195°C, which was that found in the original culture

In view of these findings we are led to suggest that Basrah III is not a chemically pure strain but is composed of two strains which differ in their specific carbohydrates, although their proteins are the same. In this view, the period of rough growth corresponds to the almost complete suppression of the galactose-containing by the glucose-containing type of vibrio. Both this strain in the rough state and Rangoon Rough (2) are vibrios with slow and scanty growth, with very rough colonies, and in both a polysaccharide whose units are glucose has been found. By picking a colony we were able to obtain a culture from Basrah III of the galactose-containing organism. It further appears that a mixed strain like this may change its 'composition' through the overgrowing of one component by the other, and this change would of course have a marked effect on its agglutination reaction with various sera. Since these experiments were carried out, Basrah III has again reverted from the rough to the original smooth-rough type of growth

It is possible that Basrah IV may also contain two types of organism, with the same protein but with different specific carbohydrates. In our first work with this strain (December 1933) we found what appeared to be arabinose, although the evidence was not entirely satisfactory. In June 1934, the strain was re-studied and the presence of glucose was demonstrated. The strain at this time was rough, whereas in the previous December it had been smooth-rough. At the same time it had become resistant to all the types of cholera bacteriophage except H and possibly B, and had accordingly shown a tendency like that of the Rangoon strains to increase its bacteriophage resistances as it became rougher

We should emphasize that the changes which we have found in Basrah III and Basrah IV, and the chemical basis for which we may have uncovered, have not occurred in strains of Indian origin so far studied. A number of these latter strains have been repeatedly analysed over a period of two and one-half years, and we have not found any irregularities in the constitution of their specific carbohydrates, nor have we found any 'mixed' strains except these two from Basrah

THE EL TOR VIBRIOS

We have already reported on the constituents of an El Tor strain (Linton, Mitra and Shrivastava, 1934*b*), and have shown that it fell into a fourth vibrio group, distinct chemically from the two groups of vibrios found in cholera, and from the group constituted by the water-vibrios, but related to both. It was of interest to continue the study of the El Tor strains, and we have accordingly analysed the following vibrios from the National Type Collection, London, which we received from Captain C L Pasricha, I M S Nos 3657, 3658 and 3659, corresponding to the Doorenbos strains 49, 67 and 20 respectively. An account of these strains, which were isolated in 1930, has been given by Doorenbos (1931)

The purified globulins of these three strains showed in alkali the curve characteristic of Protein II, in original reading, rate of decline, and final level reached. In this respect they were the same as the El Tor strain already studied. The carbohydrates of all of them showed the presence of galactose. These strains thus fall into the same group (Group IV) as the other El Tor strain, and this group is

characterized by the possession of the galactose-containing carbohydrate of the cholera vibrios of Group I and by the protein which we have found in the water-vibrios   It is possible that the anomalous position occupied by the El Tor strains (agglutinability accompanied by non-pathogenicity) may be referable to this hybrid chemical constitution   It is tempting to attribute their agglutinability to their cholera carbohydrate receptors, and their non-pathogenicity to their water-vibrio protein, but as yet this differentiation cannot be made

One further point regarding the first El Tor strain may be briefly mentioned This organism gives a pronounced rough reaction with Millon's reagent but at the same time has the same amount of specific carbohydrate as is generally found in smooth vibrio strains, that is, about 6 per cent   On agar its colonies are smooth-rough and in broth the growth is of the diffuse type, without pellicle or deposit, which is associated with smoothness   There is thus a marked discrepancy between its content of specific carbohydrate and type of growth on the one hand, and its Millon-reaction on the other   This discrepancy is not infrequent among the vibrios, although it is generally less marked, and the case of this El Tor strain shows that whatever the meaning of Millon's reaction may be it does not always indicate roughness, if by roughness is meant inagglutinability and low content of specific carbohydrate   In the previous paper of this series (Linton, Mitra and Seal, 1935) we have shown that the type of reaction to Millon's reagent may vary with the distribution of the carbohydrate within the organism   The discrepancy pointed out here may be explicable on that basis

OTHER STRAINS

In addition to the findings reported above we have studied four other vibrios and the results may be briefly described

79A   This strain, when isolated from a human source in 1931, was remarkably rough and completely inagglutinable   During the two and one-half years between the isolation and our analysis it had tended to become more and more smooth, and was found to be agglutinable at 1   2,000 with anti-cholera serum, and to have only a slightly rough element in its growth on agar , in broth it had a slight pellicle, a deposit after 24 hours' incubation, and was still rough to Millon's reagent   It has been under constant observation since its isolation, and the occurrence of the gradual shift from the rough to the smooth type of growth has been carefully observed (Vardon, 1934)

In its present state it is a Group I vibrio, having Protein I and Polysaccharide I (galactose-containing)

STRAIN E   This rough strain, upon which much of our preliminary work on vibrio carbohydrates was done (Linton and Shrivastava, 1933a), is rough to Millon's non-motile, and gives the granular type of agglutination at 1   500 dilution Its polysaccharide, as we have already reported, contains arabinose (Type II), while it has Protein I, thus placing it among the Group II vibrios

79B   This is a strain of some interest   It was isolated in India from a case of cholera, and like the Basrah strains is variable in its agglutination reaction   It is strongly hæmolytic on sheep's blood agar, and in chemical composition is the same as the El Tor strains   Protein II and Polysaccharide I   From this finding it appears that strains having the same structure as the El Tor group occur in India

Strain 505 + F type bacteriophage Strain 505, the protein and carbo hydrate content of which have already been reported upon (Linton and Shrivastava, 1933b, Linton, Mitra and Shrivastava, 1934b), was treated with F type bacterio phage, and the previously smooth agglutinable strain rendered rough in growth and to Millon's reagent and also inagglutinable It was then grown *en masse* on agar in the hope that any chemical changes which had taken place could be analysed Under these conditions of growth, however, the characteristics of roughness and non-agglutinability were lost at once and the strain reverted to its original smooth state, and the chemical analysis showed that the components were also the same as they had originally been In this instance the effect of bacteriophage was trans itory, and did not survive the mass growth A similar finding was also made with another smooth agglutinating strain (1676) which became rough and inagglutinable under the influence of combined A and F bacteriophages, and which reverted at once to the smooth type when mass growth was attempted A possible explanation may be found in the assumption that in the rough cultures there were still present considerable numbers of smooth organisms (i e , organisms containing their full complement of specific carbohydrate), and that when unrestricted growth was permitted these smooth organisms rapidly and completely outgrew their rough homologues The greater rapidity of growth of smooth forms than of rough forms has been repeatedly observed, and is widespread among bacteria in general

We may mention at this point our further experiences with the bacteriophage treatment of strains upon whose characteristics in the smooth state we have pre viously reported Strains 1617 and 2027 have been treated with eight types of bacteriophage added one by one, and with bacteriophage brews that were being prepared for therapeutic purposes , and they have been left in contact with bac teriophage for months The changes observed have included the formation of bizarre types of colonies, the appearance on the surfaces of these colonies of opaque daughter colonies, and changes in fermentation reactions and in morphology, but at the same time the agglutination titre has been very little reduced These two strains appear highly resistant to bacteriophage Both were isolated from cases of cholera We have not used other methods, such as growth on phenol agar, to enforce dissociation, as we were studying the effect of bacteriophage alone

On the other hand, Vibrio 454 was given a single treatment with A type bacterio phage in 1932 and the daughter strain has remained constantly rough and inagglu tinable since then, even when grown in mass cultures Clearly there exist among the vibrio strains those which in relation to bacteriophage are resistant and balanced (2027 and 1617), those which are non-resistant but which become balanced after being treated with bacteriophage (454), and finally those which are infected with bacteriophage and remain in an unbalanced state Of the latter, the Basrah strains appear to be examples

Discussion

The work reported in this paper has indicated that variation and dissociation in the vibrios does not have a single unitary chemical basis There appear to be at least three factors at work

1   *Loss of carbohydrate*—This was the first type of variation found and is probably the chief basis for the usual smooth-rough transitions   We have previously given examples of this change and have found that in the usual rough forms encountered there is still a certain amount of carbohydrate, the disappearance is not complete   In this way the vibrio differs from a form like the pneumococcus in which the disappearance of the capsule robs the organisms completely of its specific substance, although it leaves behind the non-specific carbohydrate common to the three types   The smooth forms of vibrio have about 6 per cent to 8 per cent (dry weight) of specific carbohydrate, while the usual rough forms have less than 2 per cent, and sometimes less than 1 per cent   However, the difference in a homologous smooth and rough pair may be less than this unless precautions are taken to keep them markedly rough or smooth, because they apparently tend to fall back from the extreme conditions toward a common centre, where they may show a chemically hardly appreciable difference in their carbohydrate content, although still distinct in the agglutination reaction

Loss of carbohydrate is, however, not the whole story in the vibrios, for as we have shown, the extremely rough Rangoon Rough (2) has about as much of this constituent as its smooth homologue, and the same relatively high percentages were also found in Basrah II which possesses the glucose-containing carbohydrate   The significance of this finding cannot be assessed until further study has been made, but it demonstrates that loss of carbohydrate may be followed by a second stage characterized by extremely rough growth and the gaining of a carbohydrate of a different structure

2   *Change in constituents*—It is clear from the work on the Rangoon strains that a vibrio may give rise to a daughter strain in which the protein and carbohydrate constituents are both different from those of the parent   Whether this change was due to a mutation in the geneticist's sense or whether it was by the chance of streaking that a colony of this 'medusa-head' type was revealed, is of course impossible to decide   In either event it is clear that from a cholera strain there has been derived a rough strain differing from the parent in having the protein constituent found in the water-vibrios and an entirely distinct type of specific carbohydrate, the units of which appear to be glucose alone   Here again we are as yet unable to suggest the meaning of this observation in terms of the æcology of the vibrio, as we are still at the beginning of observations of this sort

3   *The displacement of one chemical type by another*—In the case considered under '2' above, the two constituents of the cultures, Rangoon Rough (1) and Rangoon Rough (2), had very unequal rates of growth and the slower would not appreciably interfere with the biochemical or serological reactions of the faster   In the present case, which is illustrated by Basrah III, the two kinds of vibrios in the culture have a more nearly equal rate of reproduction   The composition of the strain is not in equilibrium, but swings sometimes rapidly and sometimes slowly from one type to the other   Specifically, the glucose-containing type of organism in Basrah III during our experiments largely replaced the galactose-containing type, but after a period of six or eight weeks the equilibrium was regained, and both types were again present in about equal numbers   During the period of rough growth, it was possible to pick a smoother colony which contained organisms of the galactose type, showing that these were still present, although in much reduced numbers, in

the culture　It is needless to point out the marked variation in serological reactions which must accompany such changes in equilibrium　An anti-serum prepared against the strain when one member was in the ascendancy might not agglutinate the strain at all when after a few weeks or months the other member had gained the upper hand　In our opinion it is possible that the aberrant strains of vibrio which vary in the agglutination reaction from time to time are of this mixed composition　As we have shown in the case of the Rangoon strains the serological reactions appear to be an expression of the underlying chemical composition What appears to be the same process of displacement is evident in the experiments in the rapid sub-culturing of the rough Rangoon strains, and is marked by the gradual disappearance and reappearance of the ' medusa-head ' colonies

As the result of the work reported here and elsewhere, we have been able to isolate and analyse three types of specific carbohydrate substances among the vibrios　These types are as follows —

Type	Constituents	Source
Type I	Galactose + an aldobionic acid consisting of galactose and glycuronic acid	In many vibrios from cholera cases
Type II	Arabinose + an aldobionic acid consisting of galactose and glycuronic acid	In a few vibrios from cholera cases, and in all the non agglutinating water-vibrios studied
Type III	Glucose only, no aldobionic acid	In a dissociant [Rangoon Rough (2)], and in two aberrant vibrios, Basrah II and Basrah III

Two types of proteins have also been distinguished on the basis of their optical activity in dilute alkali　There are accordingly at least six combinations of carbohydrate and protein possible in the vibrios, and all of these have been found　The vibrio groups are as follows —

Group I (Protein I and Specific Carbohydrate I) contains most of the vibrios isolated from clinical cholera

Group II (Protein I and Specific Carbohydrate II) contains some of the vibrios found in clinical cholera　These are, however, of somewhat less frequent occurrence than those of Group I　In its composition Group II is intermediate between the cholera vibrios of Group I and the water-vibrios of Group III

Group III (Protein II and Specific Carbohydrate II) contains the non-agglutinating water-vibrios

Group IV (Protein II and Specific Carbohydrate I) contains the El Tor strains and at least two other aberrant vibrios

Group V (Protein II and Specific Carbohydrate III) contains at present only two vibrios, the dissociant Rangoon Rough (2) and Basrah II　One of the components of the mixed strain Basrah III also belongs in this group

Group VI (Protein I and Specific Carbohydrate III)　Rangoon Recovered (*vide* Addendum) and the two Japanese strains of the ' middle ' type which we have analysed belong in this group

In regard to this classification, we may point out that its special interest lies in the inter-relationships it reveals   Thus the Group II vibrios appear intermediate between the cholera vibrios and the water-vibrios, and the El Tor vibrios are also intermediate, but related to the water-vibrios through the protein instead of through the carbohydrate constituent   It shows that strains of the characteristic composition of the El Tor vibrios are present in India, and also that the vibrios of cholera, and of water, the El Tor strains, and the dissociants form a single interlocking group

The more modern developments in immunological theory have only recently been applied to the vibrios   Since it is our hope that the chemical work reported here will eventually be correlated with and serve as the basis for the serology of these organisms, it is interesting to consider the serological study made by Yang and White (1934)   These authors conclude that roughening in the vibrios involves loss of specific carbohydrate, as we have also reported   They further state ' it seems certain that a second substance, present but masked in the smooth organism, replaces in the rough vibrio the lost smooth factor and becomes the characteristic rough receptor '   They do not, however, maintain that their analysis of the agglutination reaction of the rough vibrio is complete   Our own analysis from the chemical point of view leads us to believe that the process of roughening may be somewhat more complex than Yang and White (*loc cit*) suppose, in that, as we have shown above, more than one factor may be at work   first, loss of carbohydrate, and, second, the appearance of a new type of organism with a new type of carbohydrate   As already stated the loss of carbohydrate is in our experience the commonest accompaniment of the smooth-rough transition, and does not involve the uncovering of a second carbohydrate, nor have we ever been able (save in the apparently rare case of the ' mixed ' strains) to isolate more than one carbohydrate from a given strain   If a second type of carbohydrate of distinctive composition were present after roughening we should be unable to obtain clear-cut results in the chemical analysis, but we have on the contrary never experienced any difficulty in this respect   To us it appears probable that the ' new ' carbohydrate in the rough strain is simply the appearance brought about serologically by the incomplete disappearance of the original polysaccharide, a change which would bring about a marked variation in the serological reactions

When, as does occur, a new type of carbohydrate appears, it is accompanied by a profound change in the organism itself   It is of course possible that in the case studied by Yang and White (*loc cit*) both changes did occur simultaneously and that the variation was of the type shown by the change from Rangoon Rough (1) to Rangoon Rough (2), but the two types of change do not necessarily accompany each other, and may operate separately   The third factor in vibrio variation, that of replacement of one type by another by overgrowth, also involves the appearance, or rather the predominance of a new type of carbohydrate, but it is unlikely that the change studied by Yang and White was of this kind, since from the description their strain was a stable one

Yang and White have also likened the phenomenon of roughening in the vibrios with that in the Salmonella group and are of the opinion that the two are the same Serologically, this may be correct, but a final decision as to the actual similarity or dissimilarity must await a chemical analysis of the Salmonella group along the same lines as we have applied to the vibrio group.

SUMMARY

We have studied the chemical changes which underlie dissociation in the vibrios and have found that at least three factors are at work   The first is loss and gain of specific carbohydrate, the second the appearance in a dissociant of an entirely distinct type of protein and carbohydrate, and the third is the situation in which two types of specific carbohydrate are present in a strain, and the equilibrium between the vibrios bearing the two types is unstable   It has proved possible to correlate these changes to some extent with the serological changes which accompany dissociation, by using the method of cross-absorption of agglutinins with strains of known chemical composition

A third type of specific polysaccharide in the vibrios has been isolated and analysed   It appears to be built up of units of glucose alone and does not contain an aldobionic acid   This carbohydrate was first found in a dissociant having the 'medusa-head' type of colony and although this strain was considered to be rough it still contained as much of the polysaccharide constituent as the smooth homologue did   It appears that dissociation to this stage is accompanied not by further loss of carbohydrate but by the increased production of a different type of carbohydrate This kind of dissociation is accordingly quite distinct from that found in the usual smooth-rough transition

Daily sub-cultures of four rough inagglutinable strains in 0 5 per cent glucose broth brought about in each case a reversion to a smoother type of growth and agglutinability

Further study has been made of the El Tor vibrios and they have been found to form a chemically distinct group, although one that is closely related to both the cholera vibrios (through the specific carbohydrate), and to the water-vibrios (through the protein)   Strains of the same chemical type as the El Tor strains have also been found in India

Certain vibrios isolated from cholera have proved almost refractory to the influence of bacteriophage in that they have not dissociated to any extent either serologically or chemically after bacteriophages both singly and in various combinations had been applied to them for months

The 'Discussion' includes a consideration of the groupings which have emerged from our studies on the antigenic structure of the vibrios

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Addendum —Since this paper was sent to the press Dr S C Seal and the senior author have on two occasions isolated from Rangoon Rough (2*a*) a smooth and apparently stable strain, which is agglutinable with Rangoon Smooth anti-serum at 1 6,400, and, unlike its immediate parent, inagglutinable with Rangoon Rough (2) anti-serum This strain, which we have designated Rangoon Recovered, was resistant to types A, K and L bacteriophage, and was lysed by the other types (Vardon, 1934) Its parent strain was, as we have shown, resistant to all ten types of cholera bacteriophage

Rangoon Recovered was obtained by the method of repeated sub-cultures in 0 5 per cent glucose broth which has been described in the body of the paper Three courses of 5-day sub-culturing were used, and between each course the culture was plated and a smooth colony picked as the source of the culture for the next course

Structurally, this strain was found to contain the Type III (glucose) poly-saccharide and Protein I It thus falls into Group VI, and is the first strain of this group which we have found in India It has the same composition as the Japanese strains of the ' middle ' type, which are stated by Japanese workers to be weakly virulent

A STUDY OF VIBRIO FILTRATES

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THE work reported here began as a study of Shwartzman's phenomenon in the vibrios It has been extended as further experiments suggested themselves to neutralization of vibrio filtrates with specific anti-sera *in vitro* and *in vivo*, to the concentration of the reactive substance in the filtrates and its reactions in animals, and to some precipitin tests

The hæmorrhagic reaction which Shwartzman discovered was described by him in a series of papers beginning in 1928 He showed that the intracutaneous injection into a rabbit of 0 25 c c of a filtrate of a young culture gave in 24 hours practically no reaction If, however, this was followed by the intravenous injection after the 24-hour interval of 1 0 c c to 1 5 c c of the same filtrate, there appeared at the site of the skin injection a severe hæmorrhagic reaction which reached its maximum within a few hours and often went on to necrosis In the extended study which followed the announcement of the phenomenon, a large body of facts were brought out, and the bibliography of the Shwartzman reaction now extends to over fifty papers These cannot be adequately summarized here The actual nature of the phenomenon, and its possible relationship to Arthus' phenomenon and to anaphylaxis have been considered by Shwartzman (1930, who gives a summary of the work up to that time and a bibliography) and by Gratia and Linz (1932 , 1933) The latter workers have made the interesting suggestion that the phenomena of Shwartzman and of Sanarelli in experimental cholera in rabbits are the same

Our interest in Shwartzman's phenomenon lay in seeing how specific the reaction might be between filtrates from the various chemical groups of vibrios which we have outlined elsewhere (Linton, Shrivastava and Mitra, 1935), and in determining

whether we might gain any insight into the structure of these organisms through the study of their filtrates

The filtrates were prepared as follows Ten to twelve c c of a 24-hour broth culture of the vibrio were planted into each of 30 to 50 Roux flasks After 20 hours' incubation the heavy growth was scraped off into the broth already in the flasks No other liquid was used in washing The bacterial mass was filtered first through Kieselguhr and then through an L-3 candle It was found that filtration through Seitz E-K discs removed much of the reactive power of the filtrates The sterile water-clear filtrate was kept in the ice-box at 10°C It appeared to lose a good deal of its potency after standing about three weeks

Filtrates were prepared in this way from four organisms, representing the first four groups of vibrios which we have recognized on the basis of their chemical structure Group I, cholera vibrio 1617 , Group II, cholera vibrio 2027 , Group III, water-vibrio W 880 , Group IV, vibrio El Tor

The following is a typical experiment in the use of these filtrates for the production of Shwartzman's phenomenon 19th July, 1934 1 p m —0 25 c c of each of the filtrates (1617, 2027, W 880 and El Tor) was injected as superficially as possible into the shaven skin of a rabbit's abdomen

20th July, 1934 —The reactions at the sites of the injections are slight They consist of faint pink areas, scarcely distinguishable from the normal skin The animal is normal

11 a m (22 hours after the skin injections) —The rabbit was given 1 5 c c of W 880 filtrate intravenously

1 hour —The reaction has begun in the areas of 2027, W 880 and El Tor Area 1617 is negative The positive areas show at this time only a slight purple tinge and are not well defined

2 hours —All the areas are now definitely positive The lesions consist of purple hæmorrhagic patches which are coalescing and darkening They are not yet well enough defined to be measurable The animal has become definitely ill and is in a state of collapse Its stools have become loose

4 hours —The areas have the following dimensions 1617, 3 5 cm × 3 5 cm , 2027, 4 0 cm × 2 0 cm , W 880, 3 5 cm × 2 5 cm , El Tor, 3 0 cm × 1 0 cm These areas are now a very deep purple, raised and with glistening surfaces, they are well defined and marked off from the surrounding tissue

6 hours —The areas have not increased in size The purple areas stand out sharply as raised patches on the skin The rabbit is extremely ill and is in a state of collapse

24 hours —The reactions are about the same size as before and are well defined and raised (Plate XXIV) The two upper areas on the skin have coalesced to a slight degree The animal's skin outside the lesions is cold, shrivelled and markedly cyanotic The rabbit is still very ill and its stools are of liquid character

48 hours —The raised purple lesions are now surrounded by a dark-red band about 0 5 cm in width Otherwise they are unchanged The rabbit is definitely better and has begun to eat

72 hours —Regression has begun in all the areas, and the animal has recovered perfectly

PLATE XXIV

Shwartzman phenomenon with vibrio filtrates in the rabbit   The filtrates in amounts of 0 25 c c each were injected as follows   upper left, cholera vibrio 1617 , upper right, cholera vibrio 2027, lower left, water vibrio W 880 , lower right, vibrio El Tor (The reader's right and left are referred to)   Twenty two hours later the areas of injection were practically negative and the rabbit was then given 1 5 c c of W 880 filtrate intravenously   The hæmorrhagic reactions appeared at their full extent between four and six hours later   The picture was taken twenty four hours after the intravenous injection   The lesions are raised, well defined and dark purple with glistening surfaces The surrounding skin is clammy and cyanotic   The difference in size of reaction between the El Tor area and the other areas is well shown.   As shown in the text, this was a constant finding

It is evident at once from Table I that there is no specificity in Shwartzman's phenomenon in the vibrios   the intravenous injection of any of the filtrates brings about the hæmorrhagic reaction in all the areas   There is, however, a pronounced quantitative difference between the filtrate of the El Tor strain and the other filtrates When the El Tor filtrate is given intradermally it does not prepare the skin as well as the filtrates of the other three strains, but although the hæmorrhagic areas are always smaller they are as characteristic and as well defined as in the reactions to the other filtrates   When given intravenously, El Tor filtrate gives rise to a better reaction in the areas prepared by the other filtrates than in those prepared by itself   The average area of hæmorrhage in the four El Tor areas was $1\,7\,\mathrm{cm}^2$, in contrast to the average areas of $6\,5\,\mathrm{cm}^2$ for 1617, $6\,1\,\mathrm{cm}^2$ for 2027, and $8\,1\,\mathrm{cm}^2$ for W 880

The experience with the El Tor filtrate suggests that the filtrate used in the skin is of more importance in obtaining a large reaction than is the filtrate which is given intravenously

As we have shown elsewhere (Linton and Mitra, 1934 ; Linton, Mitra and Seal, 1935) it is possible to separate the vibrio organism into fractions, which we have designated as ' A ', ' B ' and Residue, and we have studied some of the characteristics of these fractions including the distribution of the specific polysaccharides in them and also their constitution as shown by nitrogen distribution

It was of interest to see if any one of these fractions could by itself prepare the skin for the hæmorrhagic reaction   The following experiment is typical of those that were carried out —

8th October, 1934, 11-30 a m —The following injections were made as superficially as possible into the skin of the shaven abdomen of a rabbit, in amounts of 0 25 c c each   killed heavy suspension of cholera vibrio 2027 (referred to as ' whole '), solutions of ' A ', ' B ' and Residue fractions of 2027, and filtrate of 2027 as a control

9th October, 1934, 10-30 a m —Twenty-three hours after the injections the animal was perfectly normal and all the areas were slightly pink and raised, except that into which the whole vibrios had been injected   This was practically negative

0 35 c c of 2027 filtrate was given intravenously   The reason for the small intravenous dose may be explained here   The experiments reported in the first part of this paper were done at the Pasteur Institute, Shillong, Assam, using Belgian rabbits   It was only occasionally that a dose of 1 0 c c or 1 5 c c was found to cause the death of one of these animals   The present experiments were carried out at the All-India Institute of Hygiene, Calcutta, where only albino rabbits were available   These, while nearly of the same weight as the Shillong animals, were found to be highly susceptible to the filtrates, so that in some cases doses intravenously of as little as 0 1 c c were fatal within four or five hours   When the animals did survive, however, the Shwartzman phenomenon in them was even better marked and more striking than in those in Shillong, due probably to their thinner skins and the absence of pigment   In both the Shillong and Calcutta rabbits it was observed that a less good skin reaction occurred if the rabbit was made very ill by a large intravenous dose of the filtrate, than when a moderate dose was given

1 hour —No change except in the Filtrate area, which is faintly purple   The rabbit is normal

2 hours —The Filtrate area is more markedly hæmorrhagic, and is 2 5 cm × 2 0 cm in diameter , ' A ' area is beginning to show a purple tinge which is approximately 1 0 cm × 1 0 cm in diameter   The other areas are unchanged

4 hours —The Whole area is negative , ' A ' area has a well-developed reaction, raised and deep purple, 1 0 cm × 1 0 cm in diameter   The ' B ' and Residue areas are negative , the Filtrate area is 3 0 cm × 1 5 cm in diameter —a typical reaction in size and appearance   The rabbit is in a state of collapse and has loose stools

24 hours —The Whole area is negative , the ' A ' area shows a characteristic hemorrhagic reaction, 1 5 cm × 1 0 cm in diameter   ' B ' area is negative   Residue area shows a faint redness, not the typical purple reaction   The hæmorrhage in the Filtrate area is heavy and 3 0 cm × 2 0 cm in diameter

The reactions remained about the same until 72 hours after the intravenous injection, except that the red reaction in the Residue area quickly faded Regressive changes then began   The animal completely recovered

In the experiment described above strain 2027 was used   Other experiments have been carried out using the organisms already described of the other three groups   In the case of 1617 the Filtrate area and the ' A ' area were again positive and the Whole, ' B ' and Residue areas negative   A similar finding was made when the fractions and filtrate of strain W 880 were used , in this case, however, the Whole area also showed a small hæmorrhage, which was much less marked than that of the other two positive areas, ' A ' being 1 0 cm × 1 0 cm and the Filtrate 2 0 cm × 2 0 cm in diameter and both typical   In the case of the El Tor fractions and filtrate we again found that only the areas prepared by ' A ' fraction and by the filtrate itself reacted after the intravenous injection of the El Tor filtrate

This set of experiments strongly suggests that the portion of the vibrio body which is concerned in the preparation of the skin for the hæmorrhagic reaction is the ' A ' fraction

Neutralization —In the following experiments an attempt was made to neutralize the skin sensitizing factor in the filtrates by means of specific anti-sera —

Tubes containing the following mixtures were prepared and incubated for four hours at 56°C   The dilutions indicated are final dilutions —

Tube 1   0 25 c c   undiluted filtrate 2027 + 0 25 c c   normal saline
Tube 2   0 25 c c   „   „   2027 + 0 25 c c   anti-serum 2027 (1   50)
Tube 3   0 25 c c   „   „   2027 + 0 25 c c   human convalescent serum
                                                                                                (1   50)
Tube 4   0 25 c c   „   „   2027 + 0 25 c c   normal human serum (1   50)

The homologous agglutinating titres of the 2027 anti-serum was 1   2,000 and of the human convalescent serum against this organism 1   1,000

The following were the results during the incubation period —

TABLE II

Period	Tube 1	Tube 2	Tube 3	Tube 4
15 minutes	0	Faintly cloudy	0	0
2 hours	0	Loose flocculation collecting at bottom of tube	Faintly cloudy	0
3 ,,	0	Ditto	Fine granular precipitate; no flocculum	0
4 ,,	0	Ditto	Ditto	0

As Table II shows, the homologous anti-serum began to react almost at once and at the 2-hour period a bulky loose flocculum had formed, which was easily broken up on shaking The human convalescent serum did not begin to react with the filtrate until the second hour of incubation and did not form a flocculum but a granular precipitate The control tubes 1 and 4 were negative

At the end of the four hours' incubation, the tubes were well shaken and 0 25 c c of each was injected as superficially as possible into the shaven skin of a rabbit Eighteen hours later none of the sites of injection was at all well defined Area 1 was slightly red , area 3 was represented by a small red spot and areas 2 and 4 were practically unmarked One c c of the filtrate of 2027 was given intravenously

1 hour —The animal is definitely ill Area 4 is beginning to show hæmorrhage , the other areas are negative

2 hours —Areas 1, 3 and 4 are all positive, but the hæmorrhages are not yet well defined In each of them an area 2 cm to 3 cm in diameter is covered by a mass of discrete pin-point hæmorrhages Area 2 does not have this appearance, but is represented by a single purple spot a few mm in diameter

6 hours —Area 1, 3 5 cm × 2 5 cm , 3, 3 5 cm × 1 5 cm , 4, 2 5 cm × 2 5 cm All display the typical well-marked purple raised hæmorrhagic character Area 2 is represented by a few pin-point hæmorrhages, but the skin surrounding the site of the injection does not differ from normal skin There is no general reaction in the rabbit, which is not as ill as at the 1- and 2-hour periods A diarrhœa is present

24 hours —Areas 1, 3 and 4 have the same sizes as before and are typical in appearance Area 2 is represented by a few small hæmorrhages in a clear skin This area is not raised Diarrhœa is still present

72 hours —Regressive changes are beginning in areas 1, 3 and 4 Area 2 is completely negative The animal has completely recovered

This experiment demonstrates that the hæmorrhagic phenomenon in the vibrios can be neutralized by specific vibrio anti-serum The reaction was not

neutralized by normal human serum or by saline   The failure of the convalescent human serum to neutralize cannot be taken as conclusive, since the agglutinin titre of this serum was only half that of the anti-vibrio serum which did neutralize   We have no evidence to indicate what relationship, if any, exists between the agglutinins and the neutralizing bodies in the vibrio anti-sera

This experiment was repeated using the same sera but in dilutions of 1   200 instead of 1   50   At this dilution no flocculation occurred in any of the tubes and only a slight cloudiness in the tube containing the 2027 filtrate and anti-serum   Without repeating the details of this experiment, we may say that here again the skin reaction following the intravenous injection of the homologous filtrate was averted, while the areas containing the filtrate plus saline, plus convalescent human serum and plus normal human serum were well marked and typical   The neutralization of the 2027 filtrate by its own anti-serum was, however, not as complete as when the 1   50 dilution was used, and at 48 and 72 hours the area resembled a small contusion   It is interesting to note that in this experiment the swelling was as marked in this area as in the other areas, while the hæmorrhagic reaction was almost completely absent   As before, the area became completely negative, while the other sites of injection were still well defined

Cross-neutralization was then tried, and the following experiment was set up   The dilutions are final dilutions —

Tube 1   0 25 c c   undiluted filtrate   W 880 + 0 25 c c   normal saline
Tube 2   0 25 c c   ,,   ,,   W 880 + 0 25 c c   anti-serum 2027 (1 · 50)
Tube 3   0 25 c c   ,,   ,,   1617 + 0 25 c c   normal saline
Tube 4   0 25 c c   ,,   ,,   1617 + 0 25 c c   anti-serum 2027 (1   50)

When incubated at 56°C in the water-bath, these tubes showed the following reactions at the periods indicated —

TABLE III

Period	Tube 1	Tube 2	Tube 3	Tube 4
15 minutes	0	0	0	Cloudy
2 hours	0	Very faint cloudiness	0	Uniform granular precipitate
3 ,,	0	Ditto	0	Flocculation a n d deposit
4 ,,	0	Ditto	0	Ditto

The reaction with W 880 filtrate (tube 2) did not go beyond a barely recognizable turbidity   In tube 4 the reaction of 1617 filtrate with 2027 anti-serum was visible almost at once and went on to the formation of a loose flocculum at three hours   This stage was preceded by the appearance of a granular precipitate

At the end of four hours' incubation the tubes were well shaken and 0 25 c c of each was injected into a rabbit's abdomen as before

Water-vibrio 880 is not agglutinable with the 2027 anti-serum used in this experiment (although it is agglutinable at 1 : 100 with another 2027 anti-serum having a homologous titre of 1 : 12,800), while cholera vibrio 1617 is agglutinable at 1 : 1,600.

Twenty hours after the injections, areas 1 and 2 were visible as pinkish-white ill-defined spots, and the other two areas were almost unchanged from normal. In none was the reaction at all pronounced. The rabbit was given 1 c.c. of 2027 filtrate intravenously.

1 hour.—Areas 1, 2 and 3 are beginning to show the hæmorrhagic reaction. Area 4 is negative.

3 hours.—Area 1, 2.5 cm. × 2.0 cm.; 2, 2.0 cm. × 2.0 cm.; 3, 2.5 cm. × 1.5 cm. The appearance of these areas is typically hæmorrhagic. Area 4 is represented by a faint purple spot which cannot be measured because it is too indefinite. The animal is ill.

5 hours.—The areas 1, 2 and 3 are typical and are the same size as before. Area 4 is now an irregular faint-purple patch containing a few pin-point hæmorrhages.

24 hours.—The first three areas are typical and of the same size as on the previous day. Area 4 shows a number of semi-confluent hæmorrhages in an area 2.5 cm. × 2.0 cm.

Two points are evident from this experiment: first, that 2027 anti-serum does not neutralize W 880 filtrate, and, second, that it does neutralize 1617 filtrate, although somewhat less well than it neutralizes its own filtrate, as shown in a previous experiment. In this experiment the neutralizing power of the anti-serum appears to parallel its agglutinating power.

A further experiment on neutralization was carried out as follows :—

Tube 1   0.25 c.c. of fresh normal undiluted rabbit serum + 0.25 c.c. of undiluted filtrate 2027.

This tube was incubated for four hours at 56°C and 0.25 c.c. of its contents were inoculated intracutaneously into a rabbit. During the incubation no cloudiness or flocculation appeared.

Tube 2   0.25 c.c. normal saline + 0.25 c.c. undiluted filtrate 2027.
Tube 3   0.25 c.c. anti-serum 2027 (1 : 50) + 0.25 c.c. undiluted filtrate 2027.
Tube 4   0.25 c.c. fresh normal rabbit serum (1 : 50) + 0.25 c.c. undiluted filtrate 2027.

Tubes 2, 3 and 4 were mixed, well shaken and 0.25 c.c. of each injected intradermally without previous incubation.

After 19 hours none of the areas was more than slightly distinguishable. The rabbit was given 1.0 c.c. of 2027 filtrate intravenously.

5 hours.—The animal is slightly ill. Area 1 is faintly purple. Areas 2, 3 and 4 are positive: 2, 2.5 cm. × 2.0 cm.; 3, 2.0 cm. × 1.5 cm.; 4, 2.0 cm. × 1.5 cm.

24 hours.—Area 1 is completely negative. Of the other areas, 2 and 4 show typical Shwartzman's reactions and area 3 has an atypical light-purple appearance. At 48 hours the appearances were the same in all the areas.

This experiment was designed to give at least an indication of the answers to the following questions   These may be put down categorically with the results

Does normal fresh undiluted rabbit serum neutralize the filtrate when injected after incubation ?   As area 1 shows, the reaction is neutralized under these conditions

Does neutralization occur when the normal serum is diluted 1   50 and injected without previous incubation ?   No neutralization occurs (area 4)

When the mixture of anti-serum and filtrate is injected without previous incubation, does neutralization occur ⸲   Areas 2 and 3 show that neutralization occurs under these conditions to a very slight extent only, i e , the atypical appearance of area 3 in contrast to the typical reaction in area 2 points to a certain amount of neutralization having taken place *in vivo*

Experiments on the concentration of the filtrates   These have taken two forms   repeated alcoholic precipitation of the active substance, and ammonium sulphate precipitation followed by dialysis   The concentrated filtrates were tested by skin injections alone and Shwartzman's phenomenon was not elicited, although they are capable of producing it

Alcoholic precipitation —A filtrate of 2027 was prepared as described above, 100 c c of the washings yielding 50 c c of clear filtrate   Twenty c c were reserved for experiments with unconcentrated filtrate and to the remainder was added 2 5 volumes of absolute alcohol   An immediate precipitate appeared, and after standing 18 hours in the ice-box the clear supernatant was decanted and the granular precipitate taken up in 15 c c of normal saline   Almost complete solution occurred at once, giving a golden-brown fluid, neutral to litmus and having a slight turbidity   This process of precipitation and solution was repeated, and the final precipitate dissolved in 1 5 c c of normal saline, representing a concentration of 22 5 times of the precipitable matter in the original solution, and the extraction of most of the colouring matter of the broth   Later work on larger amounts of filtrate, which we have detailed below, showed that 2 5 to 3 0 volumes of absolute alcohol precipitated the active substance, while 1 5 volumes took out the inorganic salts and left the reactive material in solution

0 25 c c of the concentrated filtrate was injected as superficially as possible into the abdomen of a rabbit and 0 25 c c of the unconcentrated filtrate was also given at the same time   Twenty hours later the site of the latter injection showed only a slight reddening, similar to that described in the experiments above   The area in which the concentrate had been injected had, on the other hand, a well-defined reaction 4 0 cm × 4 0 cm in diameter, surrounding an area 1 0 cm × 1 0 cm in diameter in which necrosis was beginning

At 48 hours the area having the unconcentrated filtrate was almost negative and the slight reaction had largely faded away   In the other area, however, the reaction was large, erythematous and swollen, and the central area of necrosis well advanced   At 96 hours the erythema had somewhat faded and the necrotic area was cicatrized, raised and indurated

This experiment shows that it is possible so to concentrate the filtrate of cholera vibrio 2027 by alcoholic precipitation that a marked skin reaction with necrosis could be obtained in the rabbit

When filtrates in volumes of 300 c c to 500 c c were being concentrated, it was found that the presence of inorganic salts could be an interfering factor, if they were precipitated along with the active portion of the filtrate   The following experiment showed that this difficulty could be overcome by fractional precipitation —

<div align="center">TABLE IV</div>

Filtrate 2027, 400 c c of a 20-hour culture prepared as described above and filtered through an L-3 candle

Amount of absolute alcohol added, in volumes	Total	Result, as shown by the appearance of a precipitate	Result of precipitin reaction with the precipitate v 2027 anti serum
1 0		*Nil*	
0 5	1 5	Heavy white precipitate	Negative
1 0	2 5	Turbidity	
0 5	3 0	Granular brown precipitate	Strong precipitin reaction
2 0	5 0	Light floccular precipitate	Negative
2 0	7 0	Turbidity , no settling	

After each addition of alcohol, except that at 2 5 volumes, the precipitate which appeared was centrifuged off, dissolved in a small amount of distilled water, and used in the precipitin test with the homologous anti-serum   As the table shows, only the precipitate which fell out with 2 5 to 3 0 volumes of alcohol contained the substance reacting with the specific anti-serum   The heavy white precipitate appearing on the addition of 1 5 volumes appeared to consist largely of the inorganic salts from the broth and agar   It was found essential to keep the reaction slightly alkaline to obtain a precipitate

The filtrate used in this experiment had been derived from growth of 2027 in broth   It was found that when the growth and preparation of the filtrate was carried out in peptone water, the differentiation was not as clear cut as in the above experiment, in that almost as much of the reactive material came down with 1 5 volumes of alcohol as with 3 0 volumes

An attempt to neutralize the skin reaction resulting from the injection of the concentrated filtrate was made   In each of three tubes equal quantities of the concentrated filtrate of 2027 and of 2027 anti-serum diluted as indicated were taken   In the fourth tube the concentrate was mixed in equal volume of normal saline

The tubes were incubated for four hours at 56°C with the results as shown in Table V —

TABLE V

Period	Tube 1 1 1	Tube 2 1 50	Tube 3 1 200	Tube 4 Normal saline filtrate
30 minutes	Cloudy	0	0	0
2 hours	Turbid	0	0	0
3 „	Heavy flocculum fills the tube	0	0	0
4 „	Ditto	0	0	0

After four hours' incubation the tubes were well shaken and 0 25 c c of each was injected into the skin of a rabbit

Nineteen hours later, area 1 is a small raised lump about 1 0 cm × 1 0 cm in diameter and hardly distinguishable in colour from the normal skin   Area 2 is likewise almost negative and is represented by a slight erythematous patch   Area 3 is 4 0 cm × 4 0 cm in diameter surrounding the site of injection, which is however not as marked as in the previous experiment, since there is no necrosis The control area 4 is also 4 0 cm × 4 0 cm in diameter, and is raised and markedly inflamed   At 48 hours area 1 has decreased to about one-half its size at 24 hours, area 2 is negative and areas 3 and 4 are still well defined, but no necrosis has taken place around the point of injection

It appears that the serum has neutralized the concentrated filtrate at dilutions of 1   1 and 1   50, but not at 1   200   The reaction at this dilution (area 3) and in the control area (area 4) are not as marked as that found with the undiluted filtrate in the previous experiment   This result may be due to the weakening effect of heating the concentrated filtrate at 56°C for four hours   Nevertheless, there is a definite contrast between the areas, which allows the conclusion that a certain amount of neutralization occurred under the conditions of the experiment

Concentration with ammonium sulphate —Fifty c c each of the filtrates of 1617 and W 880 were completely saturated with ammonium sulphate, and after standing overnight were divided into two portions, the 'precipitate' and the 'filtrate'   They were dialysed against running water followed by distilled water until only a very faint reaction for sulphates was present   They were then concentrated in vacuum at 50°C to a few c c , centrifuged, and the water-clear solutions put up against the anti-sera to 1617, 1617A, W 880 and W 880A   The filtrates and precipitates were used undiluted   The figures in parentheses refer to the

final dilutions of the anti-sera at which the reaction occurred   The readings were made after two hours at 56°C

TABLE VI

Anti sera	1617 precipitate	1617 filtrate	W 880 precipitate	W 880 filtrate
1617	Cloudy	0	+ (1 2)	—
1617A	+ (1 2)	±	+ (1 2)	—
W 880	0	—	++ (1 8)	0
W 880A	Cloudy	—	++ (1 2\	±

± = doubtful, — = not done

The anti-sera against the two ' A ' fractions were included in this experiment for the following reason   In the work previously reported on the serology of the vibrio fractions (Linton, Mitra and Seal, *loc cit*), we showed that these ' A ' anti-sera reacted only with whole vibrios and with ' A ' extracts, and not with the ' B ' and residue fractions   It appeared possible accordingly that some information might be gained as to the possibility of ' A ' being present in the filtrate, by including these anti-sera

As Table VI shows, the two precipitates react with the anti-sera (with the exception of W 880 serum against 1617 precipitate), while the reactions with the filtrates are either negative or doubtful   W 880 precipitate appears to be more reactive than 1617 precipitate, and this finding was borne out by the skin experiments detailed below   The ' A ' anti-sera do react with the precipitates and only doubtfully with the homologous filtrates, thus giving at least an indication of the character of their contents   This indication is strengthened by the previous demonstration that the ' A ' fractions are able to sensitize the skin for the Shwartzman reaction

The distribution of the reactive factors in the ammonium sulphate filtrates and precipitates was also studied by means of a skin test   0 25 c c of each of the four fluids was injected as superficially as possible into the shaven skin of a rabbit's abdomen   Twenty-four hours later readings were made with the following results 1617 precipitate, a raised and erythematous area 2 0 cm × 1 5 cm in diameter 1617 filtrate, no reaction   W 880 precipitate, a raised erythematous area 3 0 cm × 1 5 cm in diameter, with a hæmorrhagic area around the site of the injection   It is a stronger reaction than that given by the 1617 precipitate   W 880 filtrate, no reaction   The reactions with the precipitates are much stronger than with the original broth filtrates, showing that considerable concentration of the active substance had occurred

At 48 hours the reactions in the ' precipitate ' areas had increased in size and severity   The diameter of 1617 area is now 2 5 cm × 2 0 cm , and that of W 880 4 0 cm × 2 5 cm   They appear as contused erythematous and hæmorrhagic areas

of irregular outline   The skin itself is cyanotic, cold to the touch, and loose   The 'filtrate' areas are negative

At 72 hours the 'precipitate' reactions had begun to regress, but were still well defined

It is evident that the results of this skin reaction coincide with those of the *in vitro* precipitin reaction   The W 880 precipitate is stronger in both experiments than the corresponding fraction of 1617, and in all cases the reactions with the filtrates are practically negative   It seems justifiable to conclude that full saturation of the original broth filtrate with ammonium sulphate is able to carry down the reactive factors and that in this way they may be purified and concentrated

Precipitin reactions with anti filtrate serum   An anti-serum was made against the unconcentrated filtrate of cholera vibrio 2027 by intravenous injections into a rabbit   The initial dose was 0 5 c c , followed four days later by 0 75 c c and at three further intervals of four days by 1 0 c c   The total given was 4 25 c c   When bled a week after the last injection the rabbit's serum had an agglutinating titre of 1   12,800 against its homologous organism   The filtrate was accordingly at least as antigenic as the whole vibrio, and as capable of producing agglutinins   The filtrate appears to give rise to precipitins more readily than whole organisms do It has been the experience of ourselves as well as of other workers that cholera precipitins are obtainable in rabbits only after numerous injections of whole organisms, which may extend over a period of 12 or 15 weeks   The injection of the filtrate produced precipitins in about three weeks

Unconcentrated filtrate of 2027 was set up against this anti-serum, and the results are given in Table VII —

TABLE VII

Final serum dilutions	15 minutes	2 hours	4 hours
1 2	++	+++	+++
1 8	Cloudy	+	++
1 16	Slightly cloudy	Cloudy	Cloudy
1 32	0	Slightly cloudy	Slightly cloudy
1 50	0	0	0

Both the serum and the filtrate controls were negative

The reaction at four hours was evident at a dilution of 1   8 of the serum This considerable discrepancy between the agglutinating and the precipitating

power of the serum led us to suppose that the filtrate had lost its reactivity during preparation, which had included filtration through a Seitz E-K disc A second filtrate was accordingly prepared, similar to those which we had used in the production of Shwartzman's phenomenon In this case the 20-hour growth was first centrifuged in a Sharples' supercentrifuge, then filtered through Kieselguhr, followed by filtration through an L-3 candle It was then set up against the 2027 anti-serum as before, as were also similarly prepared filtrates of the other organisms The results are given in Table VIII, the readings being made after four hours at 56°C —

TABLE VIII

Final serum dilutions	2027	1617	W 880	El Tor
1 20	+ + + +	+ + +	0	+ + +
1 40	+ + + +	+ + +	0	+ +
1 80	+ + + +	+ +	0	0
1 160	+ + +	+	0	0
1 320	+	0	0	0

Both the serum and filtrate controls were negative

It is evident that the reaction in this experiment with 2027 filtrate is much better than in the previous experiment, thus indicating that the Seitz E-K disc removes much of the active portion of the filtrate The table also shows that the anti-serum is specific in relation to the filtrates, reacting with them in the way as with the whole organisms in the agglutination reaction With itself and 1617 it reacts equally well, less well with El Tor, and not at all with W 880

SUMMARY

Shwartzman's phenomenon can be produced in rabbits by filtrates of 20-hour agar cultures of vibrios representing Groups I, II, III and IV The filtrate of the vibrios from each of these groups is able to prepare the skin for the hæmorrhagic reaction which results from the subsequent intravenous injection of any of the other filtrates There is accordingly no specificity in Shwartzman's phenomenon in relation to these four kinds of vibrios The El Tor filtrate prepared the skin less well than the others, although its power of eliciting the reaction when injected intravenously was as great as that of the other filtrates

Studies on the fractions of the vibrios in relation to their power of preparing the skin for the hæmorrhagic reaction have shown that the 'A' fraction alone yields a constant and typical reaction, and that the whole vibrios, and the 'B' and Residue fractions are without sensitizing effect   This finding is another indication of the important rôle played by the 'A' fraction in the reactivity of the vibrio to its environment

The sensitizing power of the filtrate of the Group II strain (2027) was neutralized by incubating it before' inoculation with homologous anti-serum diluted 1   50, and less well at a dilution of 1   200   The filtrate was not neutralized by human convalescent serum at these dilutions   The filtrate of the water-vibrio (W 880) was not neutralized by 2027 anti-serum, while the filtrate of cholera vibrio 1617 (Group I) was neutralized, ie, no Shwartzman's phenomenon followed when 1617 filtrate which had been incubated with 2027 anti-serum was used to sensitize the skin, but a typical reaction resulted when W 880 filtrate was substituted for the 1617 filtrate

This result is of interest since it indicates that the neutralizing power of the anti-serum coincides to a considerable extent with its agglutinating power, but that the coincidence is not complete is shown by the experience with the human convalescent serum, which agglutinated an organism the filtrate of which it did not neutralize   On the other hand, the factors responsible for the Shwartzman phenomenon, both in the skin and by the intravenous route, are not specific, but are common to all the types of vibrio studied

Fresh normal rabbit serum neutralized the reaction after incubation with the filtrate , diluted 1   50 and injected without incubation it did not neutralize   When anti-serum plus filtrate is injected without incubation, neutralization occurs to a slight extent only

The concentration of the reactive portion of the filtrates was carried out both by alcohol precipitation and by full saturation with ammonium sulphate   By fractional alcoholic precipitation of broth filtrates it was found that most of the inorganic material could be removed by 1 5 volumes of alcohol, and the active substance then brought down by the addition of a further 1 5 volumes   With peptone-water filtrates this differentiation was unsuccessful, as active substance appeared in the precipitates with both these amounts of alcohol   Concentrated filtrates were found to give marked skin reactions with necrosis in rabbits, in contrast to the transitory reactions which followed the injection of the un-concentrated filtrates   The skin reaction following the injection of concentrated filtrate could be neutralized by incubating this filtrate with homologous anti-serum before injection

Complete saturation of the filtrates with ammonium sulphate led equally to the precipitation of the active substance and it can accordingly be concentrated and purified in this way   The precipitates so produced were found by precipitin reactions and by skin reactions to have practically the whole of the reactive substance in them

The intravenous injection of unconcentrated filtrate into a rabbit resulted in the rapid production of an agglutinating and precipitating anti-serum, having the same range of specificity as anti-serum to the whole vibrio

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THE IDENTIFICATION OF THE COMMON RAT-FLEAS OF INDIA

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MEDICAL workers in India often experience difficulty in identifying rat-fleas and it was felt that a simple illustrated key to the identification of the common rat-fleas of India would be of help to many workers   The present article will, it is hoped, facilitate quick identification of the common genera of Indian rat-fleas and of the species of the more important genera   In this key, the differentiation is based on simple characters which are illustrated by photomicrographs and camera-lucida drawings   The present article, which does not claim to be an exhaustive treatise on all fleas, is intended primarily as an aid to the identification of rat-fleas which a worker may come across in India

Fleas have certain well-defined characteristics   They are wingless insects parasitic on warm-blooded animals   The body is chitinized and compressed laterally The legs are well developed for jumping and are provided with claws for clinging to the fur of the host   The mouth-parts are adapted for piercing and sucking

Text-figure 1 shows an entire flea viewed laterally   The body is differentiated into head, thorax and abdomen   The different parts of the head and thorax are shown in Text-figure 2   The most anterior part of the head is known as the ' frons ' The anterior ventral portion of the head is the ' gena '   In some fleas the gena bears a comb of spines and this is known as the ' genal comb '   These spines on the gena are sometimes on the ventral margin as in the case of *Ctenocephalides* or on the posterior margin as in the case of *Leptopsylla*   The thorax is composed of three easily recognized segments, prothorax, mesothorax and metathorax, each of which carries a pair of well-developed legs   The prothorax in several genera has a comb of spines on its posterior margin and this is known as the ' pronotal comb '   Another important structure useful in the differentiation of the genera of rat-fleas is the presence of the vertical internal thickening on the lateral ventral plate of the meso-thorax (Text-figures 1 and 3A, *msp s*)

The sex of the flea is determined by an examination of the terminal portion of the abdomen   In the female the upper and lower sides of the abdomen are more or less similar, while in the male the terminal portion is tilted upwards,

the dorsal side being very different from the ventral side (Text-figure 4) In addition to this, the male can be recognized by the presence of several internal curved-up chitinous structures which are absent in the female, and by the absence of the spermatheca (Text-figure 1, *spm*)
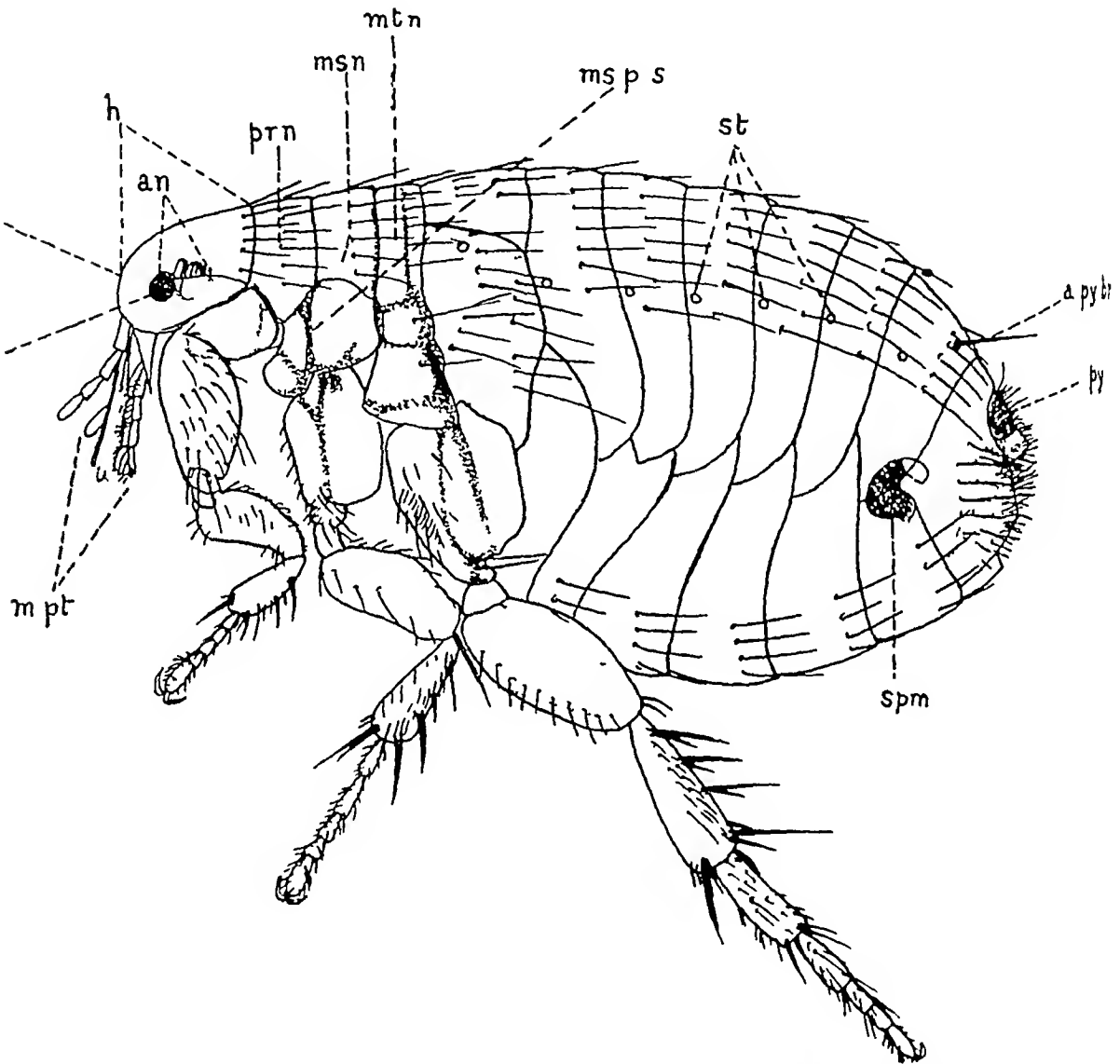
TEXT-FIGURE 1 —*Xenopsylla cheopis*, female

an antenna, *a py br* antepygidial bristle, *e* eye, *f* frons, *h* head, *msn* mesonotum, *mtn* metanotum, *m f* mouth-parts, *msp s* internal vertical thickening on mesosternite, *prn* pronotum, *py* pygidium, *spm* spermatheca, *st* stigmata.

In the identification of the genera of rat-fleas, it is important to determine the presence or absence of the genal comb and of the pronotal comb Another important character is the presence of the vertical rod-like thickening on the
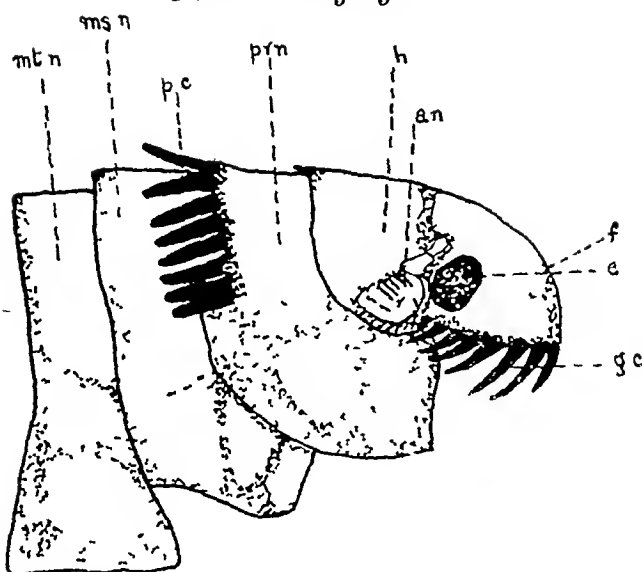
TEXT FIGURE 2 —Head and thorax of *Ctenocephalides felis orientis* (camera lucida × 70) *g c* genal comb, *p c* pronotal comb Other lettering as in Text-figure 1
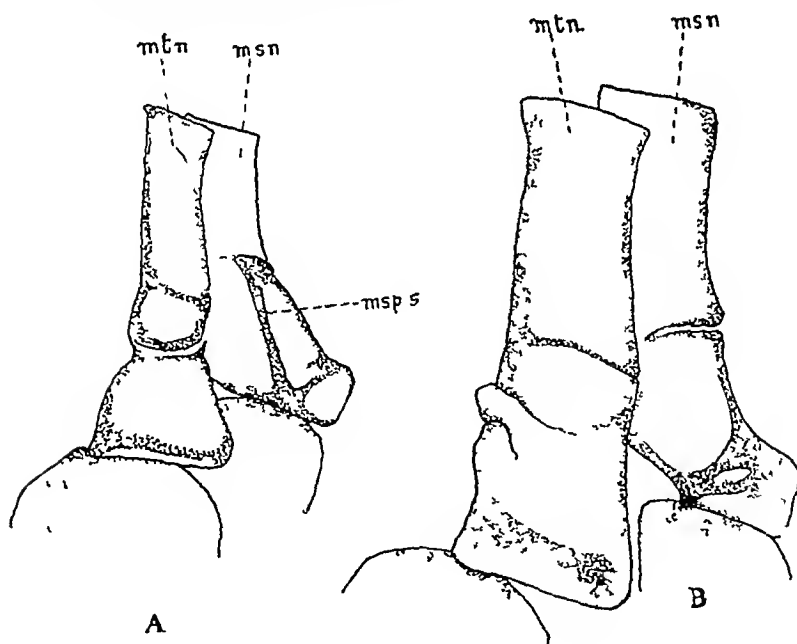
TEXT FIGURE 3 —A   Mesothorax (*msn*) and metathorax (*mtn*) of *Xenopsylla* showing internal vertical thickening (*m·p s*) on mesosternite

B   Same of *Pulex* showing absence of the vertical thickening on meso sternite (camera lucida × 95)

inner side of the pleuron of the mesothoracic segment at the point of its junction with the base of the leg In addition to these, the shape of the frons, the shape of the pygidium and the presence of eyes should be noted
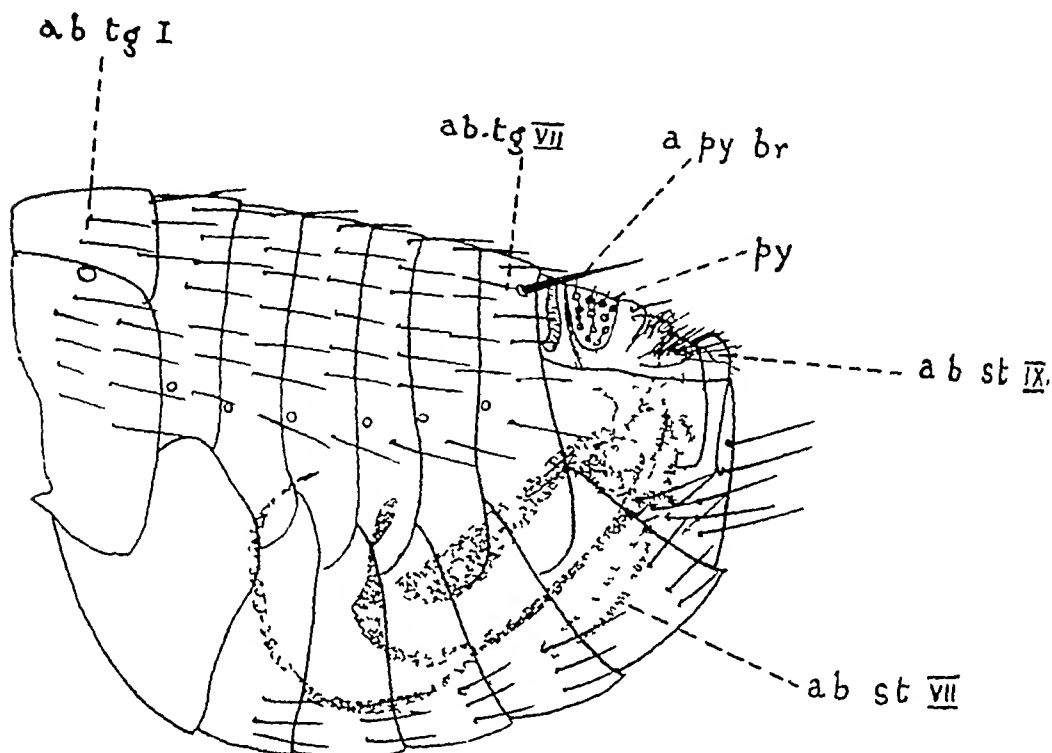
TEXT-FIGURE 4 —Abdomen of *Xenopsylla cheopis*, male
ab tg I 1st abdominal tergite, *ab tg VII* 7th abdominal tergite, *ab st VII* 7th abdominal sternite, *ab st IX* 9th abdominal sternite, *a py br* antepygidial bristle, *py* pygidium

TECHNIQUE

Fleas for identification should preferably be cleared and mounted to facilitate examination For clearing, the specimens should be dropped into a 5 to 10 per cent solution of caustic potash and kept for four hours in a water-bath at a temperature of about 80°C After this, they may be left in the same solution at room temperature overnight or even longer The specimens are then transferred to warm distilled water and washed for about two hours They are then passed through 50 per cent alcohol followed by higher grades of alcohol From absolute alcohol, they are cleared in xylol and mounted in Canada balsam

IDENTIFICATION

The following is a key to the identification of the common genera of rat-fleas of India —

1 Frons angulate, thoracic segments much shortened, thoracic tergites together much shorter than 1st abdominal segment ..   ..   ..   **Echidnophaga.**

Frons not angulate, thoracic segments not shortened,
thoracic tergites together much longer than 1st abdominal
segment   2

2 Pronotal and genal combs absent   3

Pronotal comb always present, and in some genera, genal
comb as well   4

3 Mesopleura without rod like thickening extending upwards
from insertion of base of leg, orifice of spermatheca
sub dorsal, clasper in male large and semi circular   **Pulex.**

Mesopleura with rod like thickening extending upwards
from insertion of base of leg, orifice of spermatheca
ventral, clasper in male not large or semi circular   **Xenopsylla.**

4 Eyes absent, genal comb with less than five teeth   **Leptopsylla.**

Eyes present, genal comb if present consists of more than
five teeth   5

5 Genal comb present   **Ctenocephalides.**

Genal comb absent   6

6 Dorsal margin of pygidium flat   **Ceratophyllus
                                                                                     (Nosopsyllus).**

Dorsal margin of pygidium strongly convex   **Stivalius**

Brief notes on the different genera of rat-fleas mentioned in the key are furnished
below —

Echidnophaga.

Text-figure 5 and Plate XXV, figs 1, 2 and 4)

This genus has several characteristics which at once separate it from the other
genera   The thoracic segments are much compressed and the dorsal length of the
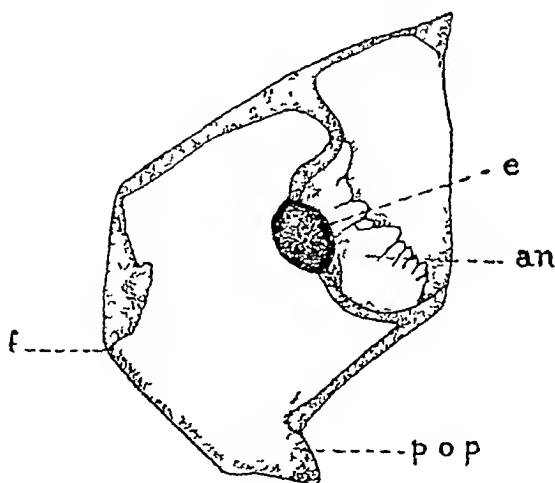
TEXT FIGURE 5 --Head of *Echidnophaga gallinaceus*
(camera lucida > 180) *f* frons, *p o p* post oral process
or genal hook

three segments together is shorter than the dorsal length of the first abdominal
segment (Plate XXV, fig 4)   Unlike other genera in which the mouth-parts

project ventrally from the head, in *Echidnophaga* the mouth-parts project more or less anteriorly   This feature is even more strikingly observed in the males of this genus   The head is polygonal in shape and the frons is angulate   The genal angle of the head is produced into a short hook-like process known as the ' post oral process' (Text-figure 5)   The legs are poorly developed   The claws are small, narrow, more or less straight and in the species found on rats devoid of the basal thumb-like process observed in other genera   The spermatheca in the female is feebly chitinized and its orifice is sub-dorsal   *E gallinaceus* Westw is a common Indian species which is normally a parasite of the fowl but frequently occur on rats as well   The female has the habit of fixing itself to the skin of the host and remaining in one position for a long time   This flea is commonly known as the ' stick-fast flea ' from the manner in which it sticks to the host   It is not easily brushed off the rat   It generally occurs on the snout of rats and also in between the toes

Pulex.

(Text-figures 3 and 6, Plate XXV, figs 5 and 6, and Plate XXVI, fig 1)

This genus is recognized by several characters   The genal and pronotal combs are absent   The mesosternum is devoid of the vertical rod-like thickening from the base of the leg to the dorsal edge of the sternal piece   The spermatheca in the female is comma-shaped and its orifice is sub-dorsal (Text-figure 6)   The upper process of the clasper in the male is large and semi-circular in shape (Plate XXV, fig 6, and Plate XXVI, fig 1)   The mandibles are broad, short and deeply serrated The hind coxa is pear-shaped
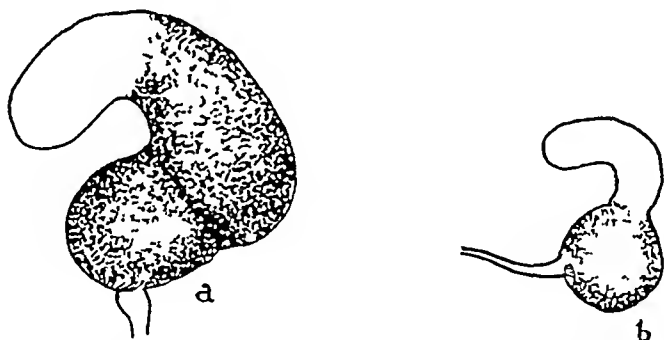
TEXT-FIGURE 6 —Spermatheca of (*a*) *Xenopsylla* and (*b*) *Pulex*, showing position of orifice (camera lucida × 193)

Pulex is represented by one species, *P irritans* L the human flea   Man is the usual host of this flea but it is occasionally found on rats   Although this flea frequently attacks rats, it is not often observed in collections of fleas off rats   This is because this flea does not ordinarily stay on the host longer than is necessary for it to have its feed of blood and usually lives away from the host   An easy method of collecting this species is to trample on dust in dark corners of rooms in areas where *P irritans* is common, when the fleas will jump on to the legs   They can then be caught in tubes containing a wad of cotton-wool wet with chloroform   Fleas collected in this manner consist largely of *P irritans*, but also include a good number of *Ctenocephalides*

PLATE XXV
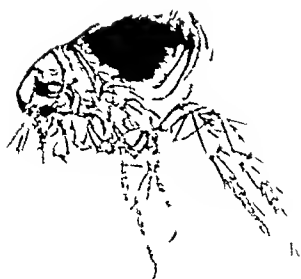
Fig 1 —*Echidnophaga gallinaceus*, male, × 30
Fig 2 —*Echidnophaga gallinaceus*, female, × 30
Fig 3 —*Xenopsylla cheopis*, showing head and thorax, × 62
Fig 4 —*Echidnophaga gallinaceus*, showing head and thorax, × 62
Fig 5 —*Pulex irritans*, showing head and thorax, × 62
Fig 6 —*Pulex irritans*, showing end of abdomen of male, × 62

PLATE XXVI
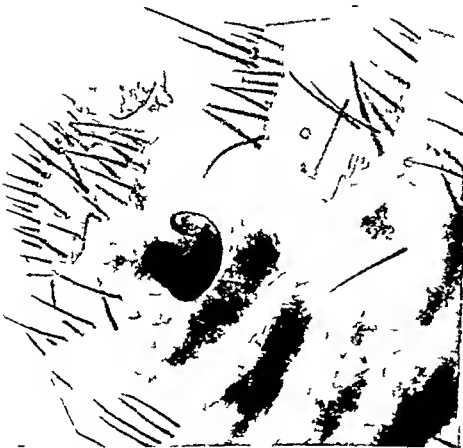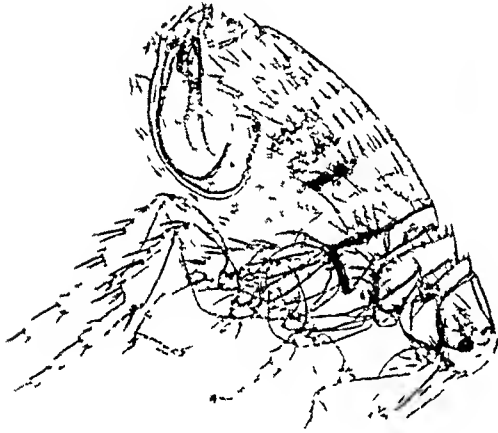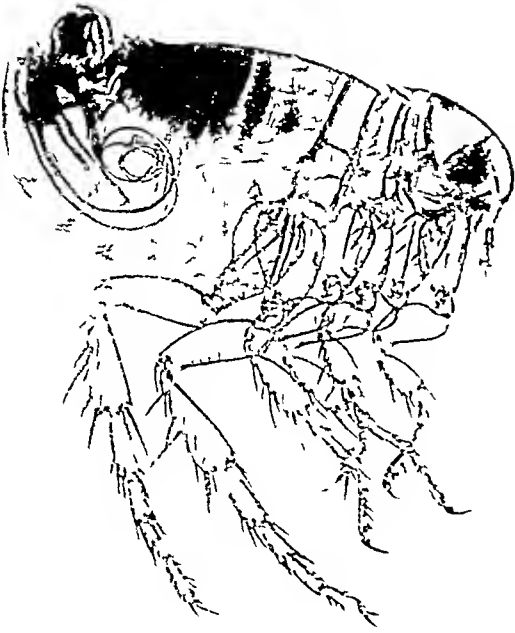
Fig 1 —*Pulex irritans*, male, × 29
Fig 2 —*Xenopsylla cheopis*, female, × 29
Fig 3 —*Xenopsylla cheopis*, male, × 29
Fig 4 —*Xenopsylla brasiliensis*, female, showing spermatheca, × 61
Fig 5 —*X cheopis*, female, showing spermatheca, × 61
Fig 6 —*X astia*, female, showing spermatheca, × 61

PLATE XXVII
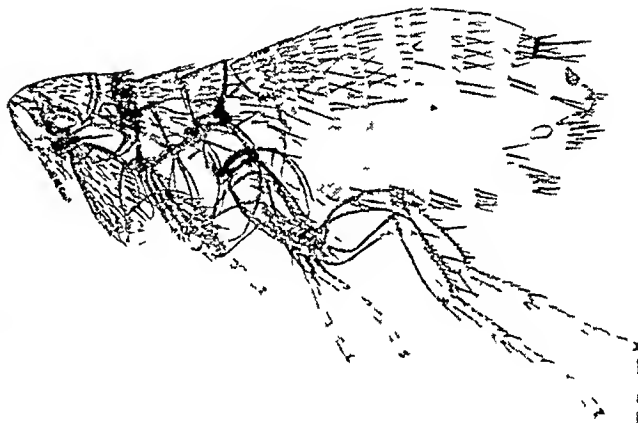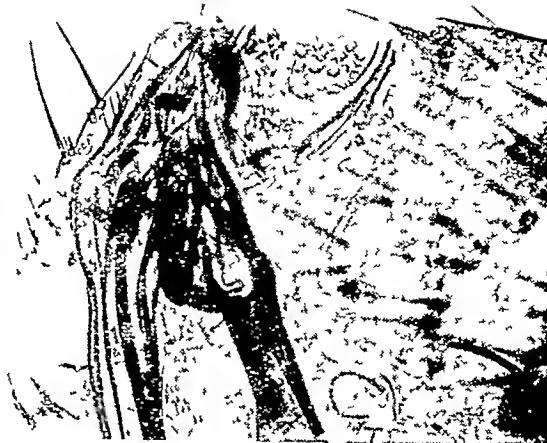
Fig 1 —Fnd of abdomen of male X *astia*, × 124
Fig 2 —Fnd of abdomen of male X *cheopis*, × 124
Fig 3 —Fnd of abdomen of male X *brasiliensis*, × 124
Fig 4 —*Leptopsylla segnis*, female, × 30
Fig 5 —*Leptopsylla segnis*, male, × 30
Fig 6 —*Leptopsylla segnis* showing head and thorax × 124

PLATE XXVIII.

3

6
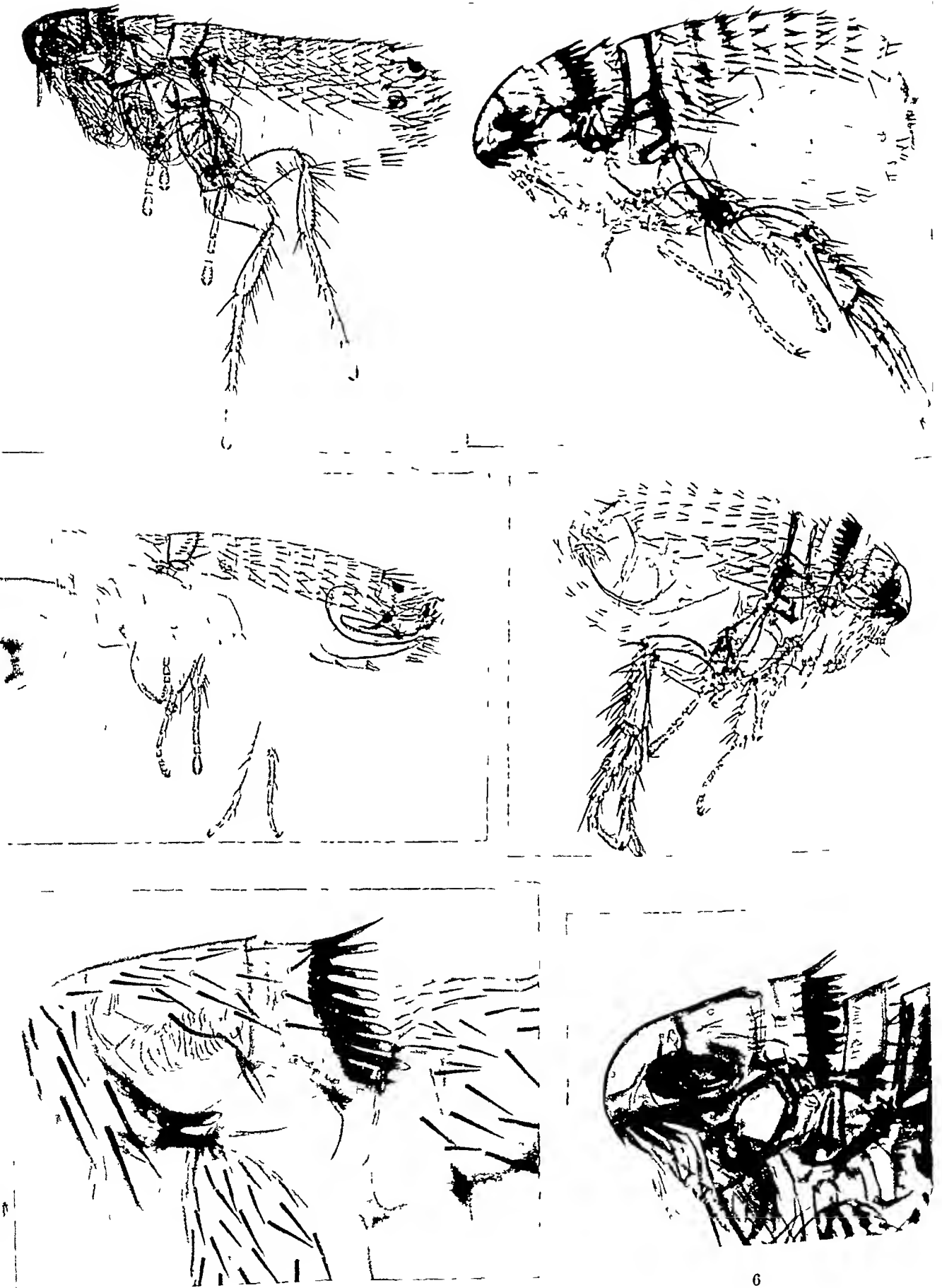
Fig 1 —*Leptopsylla himalaica*, female, ✕ 30
Fig 2 —*Leptopsylla himalaica*, male, ✕ 30
Fig 3 —*Leptopsylla himalaica*, showing head and thorax, ✕ 124
Fig 4 —*Ctenocephalides felis orientis*, female, ✕ 30
Fig 5 —*Ctenocephalides felis orientis*, male, ✕ 30
Fig 6 —*Ctenocephalides felis*,

PLATE XXIX
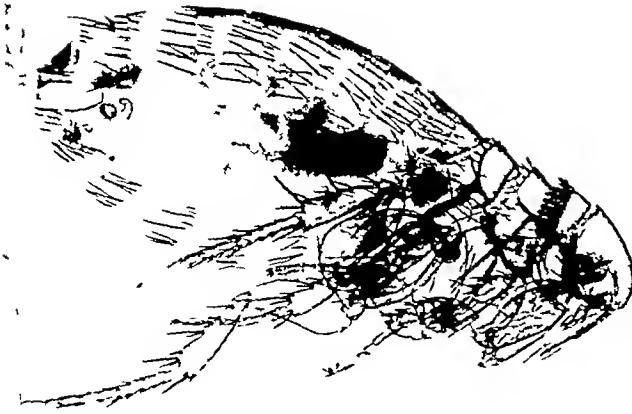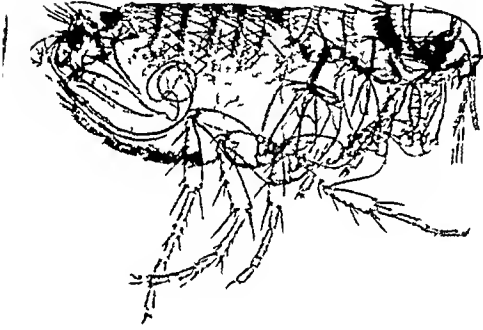
Fig 1 —*Ceratophyllus nilgiriensis*, female, × 30
Fig 2 —*Ceratophyllus nilgiriensis*, male, × 30
Fig 3 —*Ceratophyllus nilgiriensis*, showing head and thorax, × 61
Fig 4 —*Stivalius ahalæ*, end of abdomen of male showing antepygidial bristles and pygidium, × 61
Fig 5 —*Ceratophyllus nilgiriensis*, end of abdomen of female showing antepygidial bristles and pygidium, × 61
Fig 6 —*Ceratophyllus nilgiriensis*, end of abdomen of male showing antepygidial bristles and pygidium, × 62
Fig 7 – *Stivalius ahalæ* end of abdomen of female showing antepygidial bristles and pygidium, × 62

The distribution of *Pulex irritans* in India is confined to hilly areas   It is only rarely found on the plains

Xenopsylla.

(Text-figures 1, 3, 4 and 6, Plate XXV, fig 3, and Plate XXVI, figs 2 and 3)

The characteristics of this genus are   (1) absence of pronotal and genal combs, (2) presence of a vertical rod-like thickening on the inner side of the mesosternum from the base of leg to the dorsal edge of the sternal piece (Text-figure 3 and Plate XXV, fig 3), and (3) the orifice of the spermatheca is ventral (Text-figure 6) *Xenopsylla* is the principal genus of rat-fleas of India and is represented by three species, *X cheopis* Roths , *X astia* Roths , and *X brasiliensis* Baker   One of these, *X cheopis*, is the principal transmitter of plague in India and *X astia* is suspected to spread plague under certain conditions   The three species of *Xenopsylla* occurring in India can be differentiated by the following characters   (1) structure of the spermatheca in the female, (2) structure of the antepygidial bristle in the male, and (3) the shape of the ninth sternite in the male   These structures are shown in Text-figures 4 and 6

The following is a key to the identification of the females of the three Indian species of *Xenopsylla* —

    1   Head of spermatheca much broader than body (Text-
        figure 7c)   *X brasiliensis*
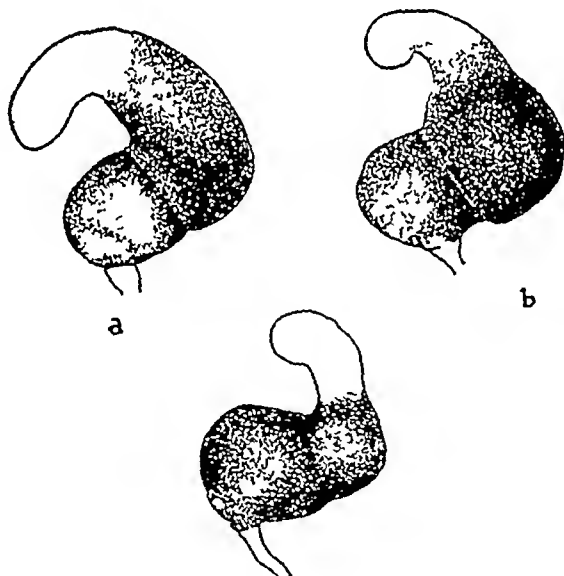
TEXT FIGURE 7 —Spermatheca of three species of
Xenopsylla (a) *X cheopis*, (b) *X astia*, (c) *X brasiliensis*
(camera lucida × 190)

    Head of spermatheca short , body much broader than
        head

2   Tail of spermatheca short, body much broader than head
    (Text figure 7*b*)   *X astia*

    Tail of spermatheca longer, body only slightly broader
    · than head (Text-figure 7*a*)   *X cheopis*

The spermatheca are illustrated in Plate XXVI, figs 4, 5 and 6

The males could be differentiated by the shape of the ninth sternite (*st ix*) and of the antepygidial bristle (*a py br*)   The following is a key to the identification of the males of *Xenopsylla* (Text-figures 8, 9 and 10, and Plate XXVII, figs 1, 2 and 3) —

1   Antepygidial bristle situated on a projecting conical
    pedicle (Text figure 8)   *X brasiliensis*   2

    Antepygidial bristle sessile without a projecting pedicle

2   Ninth sternite club shaped (Text-figure 9)   *X cheopis*

    Ninth sternite ribbon shaped or attenuated (Text-figure
    10)   *X astia*
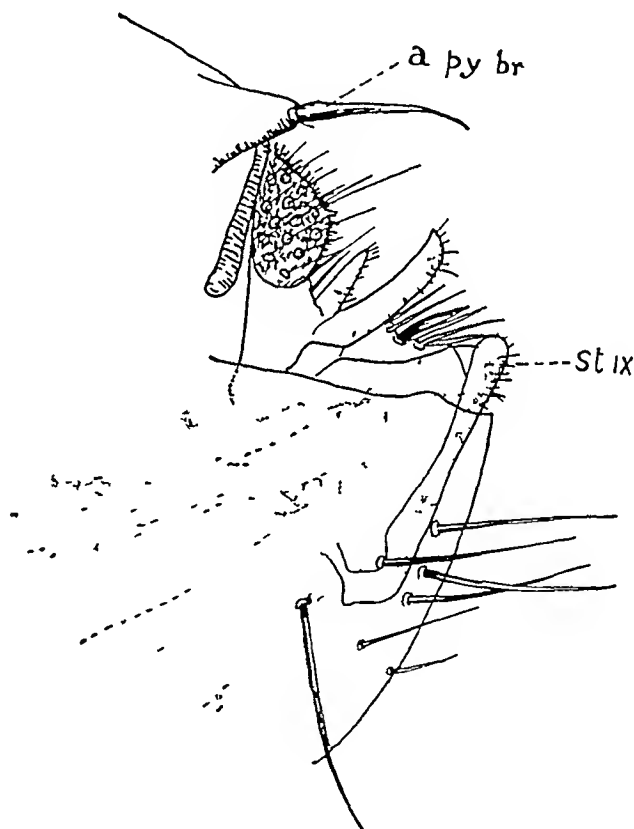
TEXT FIGURE 8 —End of abdomen of male of *X brasiliensis* (camera-lucida × 148) *a py br* antepygidial bristle, *st ix* ninth abdominal sternite

DISTRIBUTION · *X astia* occurs all over India and it is particularly common on the plains   It is less frequent in temperate zones and in elevated areas   *X cheopis* occurs principally in temperate and elevated parts of India and is less frequent on the plains   In India *X brasiliensis* is widely distributed, but it is not so numerous as the other two species
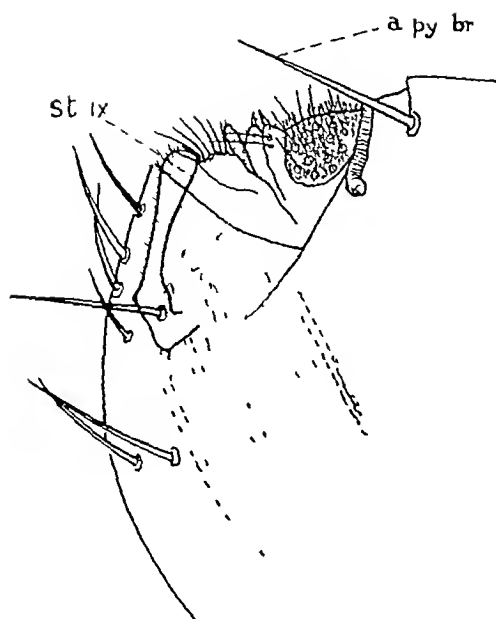
TEXT FIGURE 9 —End of abdomen of male of
X cheopis (camera lucida × 148)
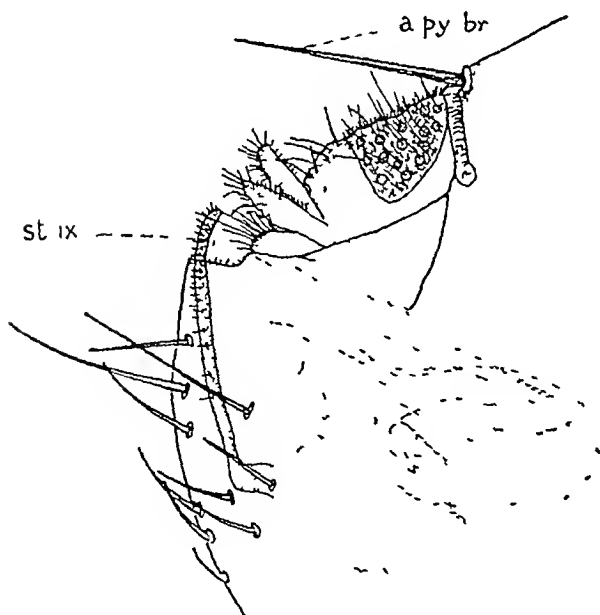
TEXT FIGURE 10 —End of abdomen of *X astia* (camera
lucida × 14S)

Leptopsylla.

(Text-figure 11, Plate XXVII, figs 4, 5 and 6, and Plate XXVIII, figs 1, 2 and 3)

This genus has several characteristic features (1) eyes are wanting, (2) the head is pointed anteriorly, (3) the genal margin is nearly vertical, while in other genera it is horizontal, (4) genal comb is present, the number of spines on the comb in the Indian species being four or less, and (5) the pronotal comb is well developed (Text-figure 11) This genus is represented in India by two species *L segnis* Schoen and *L himalaica* Roths They can be differentiated by the number of
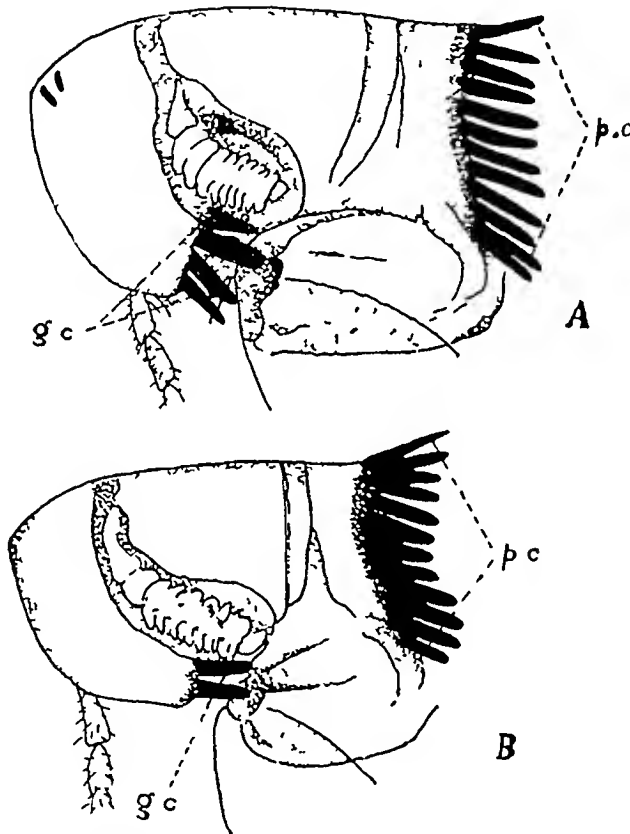
Text-figure 11 —Head and pro thoracic segment of *Leptopsylla* (camera lucida × 100) (A) *L segnis* and (B) *L himalaica* *g c* genal comb, *p c* pronotal comb

spines on the genal comb and the presence of spines at the anterior angle of the head (Text-figure 11) In *L segnis*, there are four teeth on the genal comb and there are two short spines close to the anterior angle of the head (Plate XXVII, figs 4, 5 and 6), while in *L himalaica*, the genal comb consists of only two spines and there are no spines at the anterior angle of the head (Plate XXVIII, figs 1, 2 and 3) These two species are confined to the temperate parts of India *L himalaica* has been recorded from the Himalayas, the Palni Hills and the

Travancore Hills and *L segnis* from North India and from the hills of South India

Ctenocephalides

(Text-figure 2, Plate XXVIII, figs 4, 5 and 6)

The characteristic of this genus is the presence of genal and pronotal combs, and in the species of this genus found in India the genal comb has eight teeth This genus is represented in India by three forms, namely, *C canis* Curtis, *C felis* Bouche and *C felis* subsp *orientis* Jordan   They occur on a variety of hosts and are frequently observed on rats   It must not be presumed that specimens of *Ctenocephalides* caught off dogs are *C canis* or that those caught off cats are *C felis*   The identification of the three forms is not easy and for a correct determination it is advisable to submit the specimens to an expert   A key is furnished below for the identification of the three forms of *Ctenocephalides* occurring on rats in India —

1   Most anterior genal spine much smaller than the one next
to it, hind tibia usually with seven dorsal notches,
abdominal stigmata 2 to 7, larger than socket of
antepygidial bristle   *C canis*

   Most anterior genal spine nearly as strong as the one next
to it, hind tibia with 6 dorsal notches, abdominal stig
mata 2 to 7 smaller than socket of antepygidial bristle   2

2   Frons elongate and pointed at anterior end   *C felis*

   Frons short and broadly rounded anteriorly   *C felis* subsp *orientis*

C felis orientis is the commonest form of *Ctenocephalides* occurring in India and is widely distributed   *C felis* is less common in India and occurs on the plains   *C canis* is not common and occurs in temperate areas and on hills

Ceratophyllus (Nosopsyllus)

(Plate XXIX, figs 1, 2, 3, 5 and 6),

The genus *Ceratophyllus* has recently been split up into several genera and the species occurring on rats in India fall under the genus *Nosopsyllus*   The characteristics of this genus are (1)   presence of pronotal comb (Plate XXIX, fig 3) (2) absence of the genal comb, (3) presence of three antepygidial bristles one of which is considerably reduced in the male (Plate XXIX, figs 5 and 6), and (4) the pygidium with a flat dorsal margin   Eight species of *Ceratophyllus (Nosopsyllus)* have been recorded from India, namely, *N nilgiriensis* J and R, *N punjabensis* J and R, *N tamilanus* J and R, *N simla* J and R, *N arcotus* J and R, *N argutus* J and R, *N alladinis* Roths, and *N punensis* J and R   All these species are very closely allied to one another and require expert knowledge for differentiation

Stivalius

(Plate XXIX, figs 4 and 7)

This genus has the following characteristics   (1) presence of a well-developed pronotal comb, (2) absence of a genal comb, (3) presence of three antepygidial bristles in the female and two in the male, and (4) dorsal margin of pygidium strongly convex (Plate XXIX, figs 4 and 7)   Three species of

Stivalius occur in India, namely, *S aporus* J and R , *S ferinus* Roths , and *S ahalæ* Roths   The commonest species is *Stivalius ahalæ*

The identification of the different species of *Stivalius* and *Ceratophyllus* is by no means easy and specimens are best sent to the expert on the group for determination   Both the genera are confined to the hilly and temperate parts of India *Stivalius* is fairly common at elevations over 1,500 feet above sea-level   It may rarely occur at elevations of 1,000 feet but not lower than that level

The writer is much indebted to Dr Karl Jordan, F R S , for kind criticism of the article

WAVES OF RHYTHMIC CONTRACTIONS AND RELAXATIONS IN THE PERFUSION PRESSURE TRACINGS OF THE BLOOD VESSELS OF THE FROG (*RANA TIGRINA*), WITH SPECIAL REFERENCE TO THE ACTION OF ADRENALINE, ERGOTOXINE, PITUITRIN, BARIUM CHLORIDE, JANUS GREEN AND EPHEDRINE *

BY

S A RAHMAN,

AND

R N ABHYANKAR

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WAVES of contraction and relaxation were found by McDowall (1921) to occur frequently in young and robust rabbits and cats during perfusion of the pulmonary circulation with Ringer-Locke fluid (without bicarbonate) The waves were not always regular and in different animals varied from one or two to ten per minute Pituitary extract and adrenaline stimulated the effect Such waves were also found by him in the systemic circulation

Similar waves in the perfusion pressure tracings were observed in the case of rabbits by Sharpey-Schafer and Lim (1919) as a result of the action of adrenaline These waves were obtained in the case of only some of the fifty-eight perfusion experiments performed by them Cow (1911), in his experiments on the action of adrenaline on arterial rings, occasionally obtained rhythmic contractions of the rings which showed themselves as waves upon the main curve of contraction

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* Paper read at the 22nd Session of the Indian Science Congress, Calcutta, January 1935

Similar rhythmic contractions were recorded by A J Campbell (1911) as occurring in both the pulmonary and the systemic arteries of cat and dog

Doubt has been cast on the interpretation of these results by the work of Ettinger (1932a) who, using similar apparatus to that used by Campbell, Sharpey Schafer and Lim, and McDowall, found similar rhythmic waves in the perfusion pressure tracings These waves were, however, produced artificially, for when the perfusion apparatus was used without the animal, the inflow cannula being connected directly with the outflow, identical rhythmic waves appeared on the record These waves were found to be caused by bubbles of air travelling through the glass spirals through which the perfusion fluid was conducted

By employing a slightly modified technique, we have found that waves of rhythmic contractions and relaxations are always present in the perfusion pressure tracings of the vascular system of the frog Altogether seventy-five frogs were used in our experiments

METHOD

The outlets of two bottles of convenient sizes were connected to the two upper limbs of a Y-tube by means of pressure rubber-tubing The larger of the two bottles was filled with Bayliss' solution (Bayliss, 1924) without glucose The solution was made in tap water which was found by us (Rahman and Abhyankar, 1932) to be better than distilled water for perfusion experiments on the frog's heart The pH of the solution was found to be 8 0 The smaller bottle was filled with a solution of the drug (made in Bayliss' solution) the effect of whose perfusion was to be investigated Arrangements were made to stop the flow from any of these bottles when not required All solutions were filtered through thick filter-paper before use The third limb of the Y-tube was connected to a T-tube by means of a short soft rubber-tubing, the flow through which was regulated by means of a screw clamp The other two limbs of the T-tube were connected , one with the perfusion cannula by means of soft rubber-tubing, and the other, by means of pressure tubing, to a water manometer of five-millimetre bore and to a membrane manometer which recorded on a slowly moving drum

The experiments were performed on the big Indian frogs (*Rana tigrina*) weighing on an average 0 3 kilo The frogs were killed by pithing The heart was exposed A ligature was applied to the right branch of the truncus arteriosus and also to the left branch close to the heart A cut was made into the left branch distal to the ligature and the perfusion cannula was introduced in such a manner as to reach the middle compartment of the artery The size of the cannula was of some importance , it was as large as could safely be inserted The perfusion fluid was allowed to escape through another cannula inserted into the ventricle through a slit at the apex and was delivered into the cup of a Condon's magnetic tipper which registered each fill of the cup by an electromagnetic signal

Arrangements were made to keep the pressure levels in the perfusion bottles equal and constant, being about 30 centimetres higher than the position of the frog The fluid from the larger bottle was then allowed to flow through the frog's vessels and the screw clamp, already mentioned, was gradually tightened The result of this was to impede the free flow of the fluid from the perfusion bottle with the

consequent fall of the level in the water manometer   The screw clamp was tightened only so far as to bring the level of the fluid in the manometer 15 cm above the position of the frog   The level in the water manometer indicated the pressure with which the fluid entered the perfusion cannula   The introduction of the screw clamp made the mechanism highly sensitive to any alteration of flow through the vessels of the frog   Any change in the rate of flow was registered on the drum by the alteration in the level of the writing point of the lever of the membrane manometer and also, less accurately, by the alteration in the rate of outflow into the cup of the Condon's magnetic tipper   That the resistance to the flow of the fluid was offered actually by the blood vessels and not by the perfusion cannula was ascertained on several occasions by cutting the dorsal aorta which resulted in the sudden fall of the level in the water manometer

Perfusion with Bayliss' solution

Tracings obtained on perfusion with Bayliss' solution is this manner always showed a wavy character (Text-figure 1)   The waves were undoubtedly caused by rhythmic contractions either of the blood vessels or of the viscera, since when the perfusion cannula was connected directly with the outflow cannula and the resistance to the outflow adjusted to be the same as was offered by the blood vessels of the frog, the writing lever recorded a perfectly straight horizontal line

The waves averaged about two to three per minute, the biggest waves corresponding to a pressure difference of about 0 5 cm of water   The rhythmic contractions of the blood vessels, in case the waves were taken to be caused by the contractions of the blood vessels, could only be myogenic in origin, since they were still present after the sympathetic constrictor endings were paralysed by perfusing the vessels with a solution of ergotoxine

More marked waves were obtained when the perfusion was made through the vessels of the hind limbs only, the perfusion cannula having been introduced into the dorsal aorta just above its bifurcation and the fluid drained out through the anterior abdominal vein, the renal portal veins having been ligatured   These waves were more uniform in shape and were characterized by sudden rise, amounting usually to a pressure difference of 1 0 cm or more, followed by a sudden fall, which gave the peaks of the waves a pointed appearance

Perfusion with adrenaline

The effect of this drug was studied by injecting 1 0 c c of a 0 002 per cent solution of adrenaline chloride (Parke Davis and Co) into the rubber-tubing attached to the perfusion cannula   The drug became further diluted by mixing with the perfusion fluid, the amount of dilution depending on the rate of flow   Twenty frogs were used for this purpose   The injection of adrenaline always caused an immediate and sharp rise in the tracing with a tendency for the waves to become obliterated

Perfusion of the vessels with solutions of different dilutions of adrenaline, viz , one in a hundred thousand, one in a million, and one in ten millions, gave similar results , there was an immediate rise of the writing point of the lever with the

obliteration of the waves and these remained obliterated so long as the writing point remained high   After a short time, however, the pressure began to fall, although the perfusion with the adrenaline solution was continued   As the pressure fell the waves began to reappear (Text-figure 1)   This was seen in the case of all the four experiments performed

That the action of adrenaline in obliterating the waves is independent of its constrictor action on the blood vessels is shown by the fact that, after ergotoxine
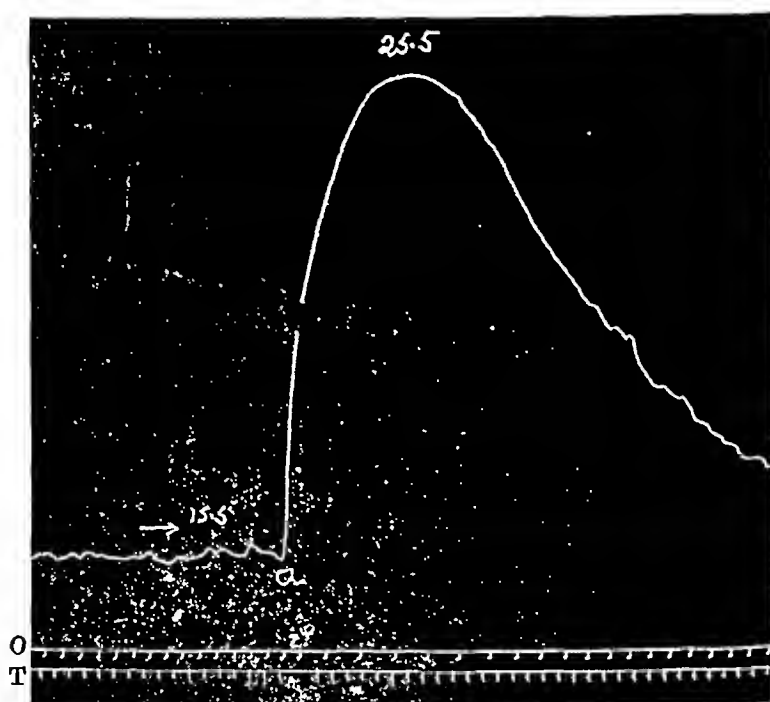
TEXT-FIGURE 1 —Perfusion through the systemic vessels of frog
Up to the mark *a*, the vessels were perfused with the Bayliss' solution   The tracing shows a well-marked wavy character At *a*, the perfusion with the adrenaline solution (1/1,000,000) was started and was continued throughout the rest of the experiment The pressure rose from 15 5 cm to a maximum of 25 5 cm   It was maintained at this level for a short period and then began to fall   At the same time the waves were obliterated   They began to reappear at the lower portion of the decline   O = output record, each mark indicating 8 c c   T = time in ½ minutes

perfusion, adrenaline injection loses its pressor action, but its action of obliterating the waves is retained (Text-figure 4)

The cause of the gradual fall in pressure could not be explained, but, as the outflow also increased simultaneously, it was evident that the fall of pressure was due to the dilatation of the vessels   Why the vessels should not respond to the continuous perfusion with adrenaline was not evident   It might have been due to the fatigue of the vessels, though the vessels responded sharply to the slightest increase in the concentration of the adrenaline solution   It is also possible that the vessels respond only to any sudden change in the strength of the drug and

develop a tachyphylactic reaction to a continuous perfusion with solution of a constant strength

Thus the effect of adrenaline is to obliterate the waves   This is in marked contrast to the results obtained by Sharpey-Schafer and Lim (*loc cit*) who found such waves in the pulmonary pressure tracings, in the case of some of their rabbits, only as a result of adrenaline injection

Perfusion with barium chloride

One c c of 1 0 per cent barium chloride solution was injected as before in the case of ten frogs   In all the cases there was a marked rise in the tracing, the
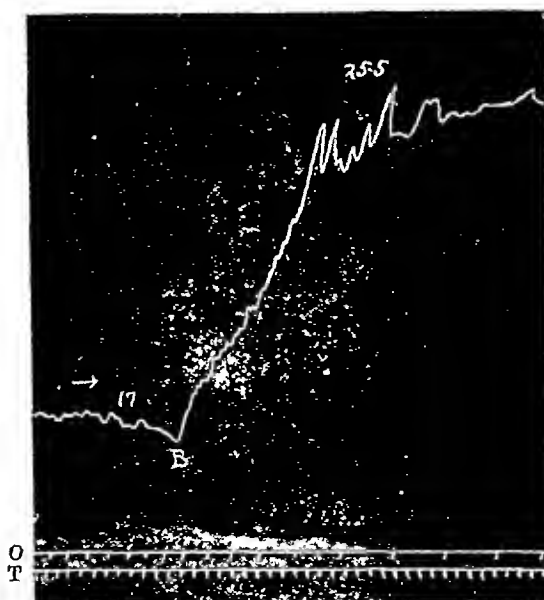
TEXT FIGURE 2 —Perfusion through the systemic vessels of frog   Up to the mark *B*, the vessels were perfused with the Bayliss solution   At *B*, the perfusion with the 1 in 2,000 solution of barium chloride was started   The tracing shows an exaggeration of the waves manifest at the top of the tracing   O = output record, each mark indi cating 8 c c   T = time in ½ minutes

characteristic feature being a definite exaggeration in the amplitude of the waves This became still more manifest when the vessels were perfused with a 1 in 2,000 solution of barium chloride (Text-figure 2)

Barium chloride acts directly on the arterial muscle   This was shown by Dixon and Halliburton (1910) and by Cow (*loc cit*)   In the case of eight of our experiments we found that paralysing the sympathetic constrictor did not interfere with the action of barium chloride   The vessels were perfused with a 0 1 per cent

solution of ergotoxine for fifteen minutes   Subsequent injection of adrenaline had no pressor effect, whereas the injection of barium chloride solution resulted in a marked rise of the lever with the exaggeration of the waves

Text-figure 3 shows the result of perfusion with a weak solution of barium chloride (1/10,000) through the vessels of the hind limbs   Up to the mark *BP*, the vessels were perfused with Bayliss' solution only   The tracing shows the usual waves   At *BP*, perfusion with the barium chloride solution was started   The solution was so dilute that there was only a slight rise in pressure   The waves,
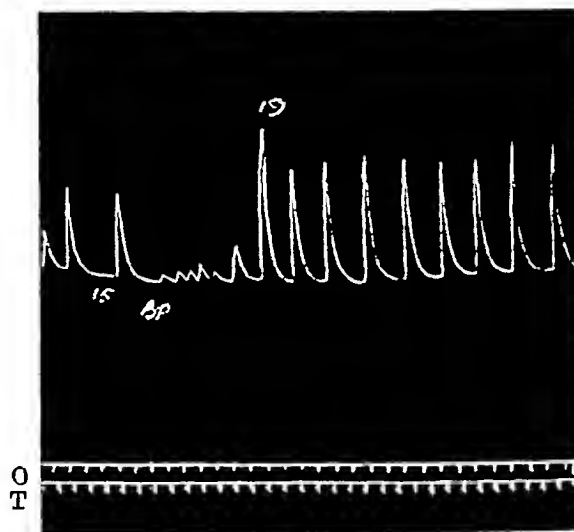
TEXT FIGURE   3 —Perfusion   through   the vessels of the hind limbs of frog   Up to the mark *BP*, the vessels were perfused with the Bayliss' solution   The tracing shows the usual waves   At *BP*, the perfusion with a dilute solution of barium chloride was started, result-ing in a slight rise of pressure but a well-marked exaggeration of waves   O = output, each mark representing 8 c c   T = time in ½ minutes

however, became definitely exaggerated both in amplitude and frequency   Such effect was seen in all the five perfusion experiments performed

Perfusion with ergotoxine and ephedrine

The effect of ergotoxine was tried in nine of the experiments   In all the cases the effect showed itself by a rise in the perfusion pressure   The effect on the waves was a remarkable increase in amplitude as well as in frequency

Text-figure 4 shows the effect of the perfusion of the vessels of the hind limbs of frog with a 0 002 per cent solution of ergotoxine ethane sulphonate (Burroughs Wellcome and Company)   The waves are seen to be frequent and their amplitude increased   At *a*, 1 c c of 0 002 per cent adrenaline solution was injected   The

perfusion pressure, instead of rising steeply, actually fell, proving that the vaso-constrictor nerve-endings were paralysed with ergotoxine   The tracing also brings out clearly the obliterating effect of adrenaline on the waves   The waves, however, begin to reappear after the effect of adrenaline is worn off   At a_1, the perfusion with 0 1 per cent ephedrine (British Drug Houses, Limited) was started, resulting in a considerable rise of pressure and an increase in the frequency of the waves Such rise of pressure was also seen in the cases where the vessels were perfused with a solution containing both ergotoxine and ephedrine
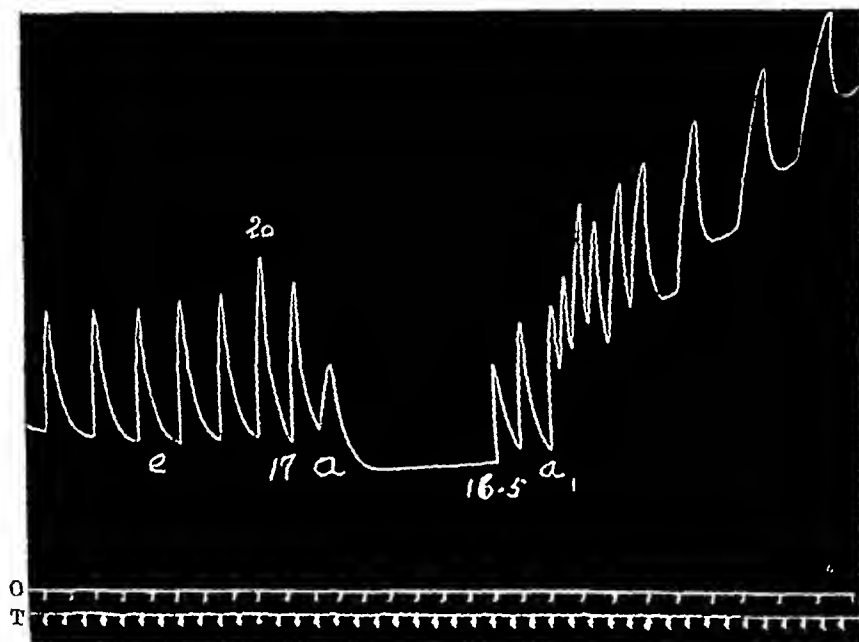
TEXT FIGURE 4 —Perfusion through the vessels of the hind limbs of frog   Up to the mark a, the vessels were perfused with a 0 002 per cent solution of ergotoxine ethane sulphonate   The tracing shows well marked waves   At a_1, 1 c c adrenaline (1/50,000) was injected   At a, the perfusion with ephedrine (1/1 000) was started, resulting in the rise of pressure and the increase in the frequency of the waves

Ephedrine, therefore, acts directly on the muscle fibres of the blood vessels and, in this respect, its action is different from that of adrenaline   These results are in conformity with those obtained by de Eds and Butt (1927)

Perfusion with pituitrin

This drug also acts directly on the musculature of the blood vessels as its effect is maintained after the sympathetic constrictor endings are paralysed by ergotoxine perfusion   This was seen to be the case in one of our experiments

The effect of pituitrin perfusion on the perfusion waves was variable. In those experiments in which the fluid entered through the aortic arch, there was hardly any effect on the waves. In the experiments where only the hind limbs were perfused the effect was to increase the frequency of the waves in the initial stages without, however, any tendency to exaggerate their amplitude (Text-figure 5)
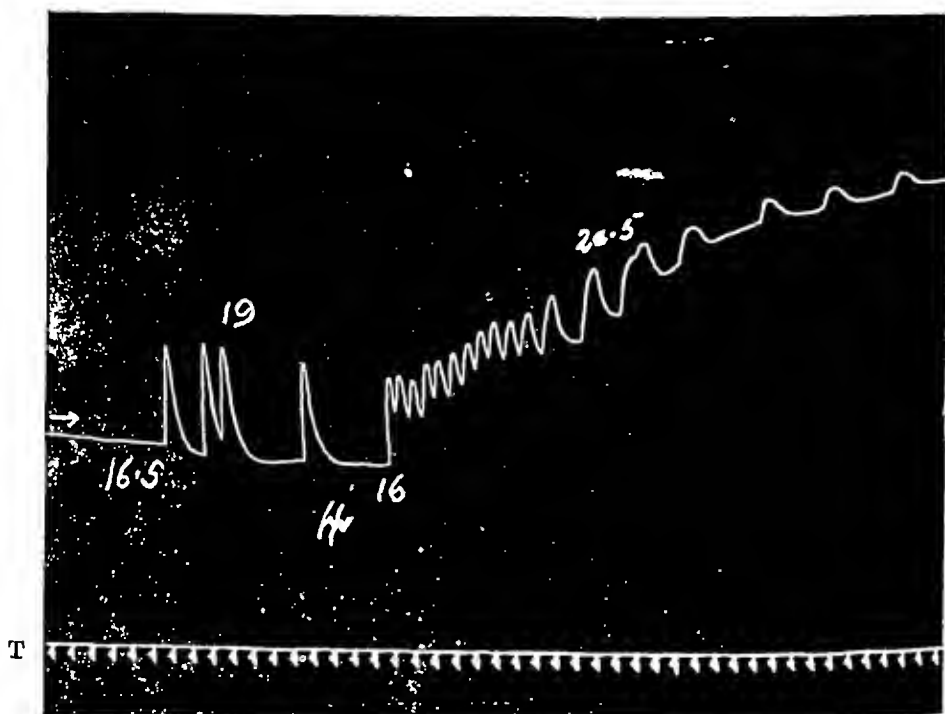
TEXT-FIGURE 5 —Perfusion through the vessels of the hind limbs of frog The waves are well marked At *pp*, the perfusion with a 0 3 per cent solution of pituitrin (Bengal Chemical & Pharmaceutical Works, Ltd) was started There was a rise in pressure and increase in the frequency of the waves   T = time in ½ minutes,

Perfusion with Janus green

The pressor action of this drug was discovered by accident by Ettinger (1932*b*)   The Janus green used by him was Janus green B (Hoechst), Janus green G (Hoechst) and Janus green B (Grubler)   There was no difference in their action in the experiments carried out by him   Dr G Grubler & Co 's Janus green was used in our experiments on ten frogs   An injection of one c c of 0 01 per cent solution had no effect   The pressor action could still be obtained after the sympathetic pressor endings were paralysed by perfusion with a solution of ergotoxine Here the action of Janus green resembled that of barium chloride with the exception, however, that there was no appreciable alteration in the character of the waves in the tracing

A second injection of Janus green, after the lever had come down to the normal position, was followed by a similar rise in the tracing   The dilator effect was not present, nor was the tachyphylactic effect manifest as described by Ettinger (1932*b*)   The perfusion with a 0 01 per cent solution of Janus green caused a

gradual rise of the lever, the vaso-constriction lasting all the time the perfusion was kept up (half an hour or more), till the results were complicated by œdema

A 0 002 per cent solution of Janus green caused a small rise in the tracing which kept up as long as the perfusion was continued   Altogether five perfusion experiments were performed with Janus green with the same results   These results were entirely at variance with those obtained by Ettinger who found that the perfusion with a solution of Janus green (1/10,000) caused a transient constriction followed by an increased rate of flow which might be as much as fivefold

These differences in the result might be due to the different brand of Janus green used by us

SUMMARY

A method is described by which the recording system becomes highly sensitive to the changes of pressure in the perfusion fluid

In some of the experiments the perfusion fluid was made to enter the left systemic arch and, after having passed through the vascular system, was allowed to escape through a cut at the apex of the ventricle   In the other experiments the posterior extremities alone were perfused, the fluid entering the abdominal aorta and, after passing through the vessels of the legs, escaping through the anterior abdominal vein   In both the cases the perfusion pressure tracings showed a wavy character

On the face of it, the waves seem to be caused by the rhythmic contractions and relaxations of the blood vessels of the frog   But other influences such as movements of the viscera are also discussed as possible factors in causing the waves, or in modifying their character   These points are still under investigation

Adrenaline tends to obliterate the waves, ergotoxine, barium chloride and ephedrine exaggerate the waves, whereas Janus green has no effect on them   The action of pituitrin is variable   The nature of the action of some of these drugs is also discussed

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A MODIFIED METHOD OF ESTIMATING ARSENIC-CONTENT OF INDIAN FOOD-STUFFS

BY

A C BOSE, PH D (Liv), A I C

AFTER the report by Bang (1902) on arsenical poisoning, Chapman (1926) has collected the available references and made a further study on arsenic-content of various English food-materials Heller (1928) has collected no less than 52 references concerning various methods of arsenic determination   More recently Gangl and Sánchez (1934) have described a method of its estimation by dissolving the arsenic-mirror in iodine-monochloride and titrating the liberated iodine in presence of cyanide and HCl (1 1), with iodate solution

Since the discovery of arsenic in food-stuffs, the whole chemico-legal aspect of accurately judging the suspected cases of arsenic poisoning has been rendered disputable

A small amount of arsenic is known to have a stimulating effect on the growth of plants (Stewart, 1922) and animals, and moreover food-stuffs, such as sea fishes, prawns, etc , having a high arsenic-content, can be consumed with impunity without the least fear of arsenical poisoning   It appears, therefore, that the organo-arsenic-complexes in these and other similar materials are present in less toxic or in harmless form   Again, La Wall and Harrison (1934) have shown that cartilaginous substances are known whose arsenic-content is greater than the tolerance for arsenic in food-products   It is apparent, therefore, that the total arsenic-content can never be accepted as a true measure of toxicity , the manner in which arsenic is present in combination and its *toxicity* should also be investigated simultaneously

In the present investigation an attempt to ascertain the arsenic-content of various Indian food-stuffs has been made by Chapman's simple method

Arsenic-free hydrogen in cylinders was not available, therefore it had to be prepared in the laboratory and with a gas-holderful of hydrogen so prepared only three to four estimations were possible   Chapman's method had, therefore, to be modified, effecting considerable economy of hydrogen, thus making 80 or more determinations possible with the same supply of hydrogen

The apparatus consists of a test-tube (5 in × 1⅜ in) fitted with a stopper with four holes, the first containing the hydrogen-inlet (H) , the second, a short-stemmed small tap or cup-funnel (C), the third, an acute-angled bent-tube (SM) , and the fourth, a tube bent at right-angle (D) (*see* Text-figure)

Procedure —Connect M with the reservoir (R_1)   Put 7 to 8 grammes of arsenic free zinc into the test-tube and let in distilled water from R_1 until the test-tube is completely filled with water   Turn off T   Now, D is closed and H connected with the gas-holder   Disconnect M and let in hydrogen slowly when the water is all forced out, screw up S   Open T, the water in the stem is pushed up due to the pressure exerted by hydrogen   Close T, pipette the water off from the cup (C) and put a few c c HCl (50 to 70 per cent) into it   Now open D and force the
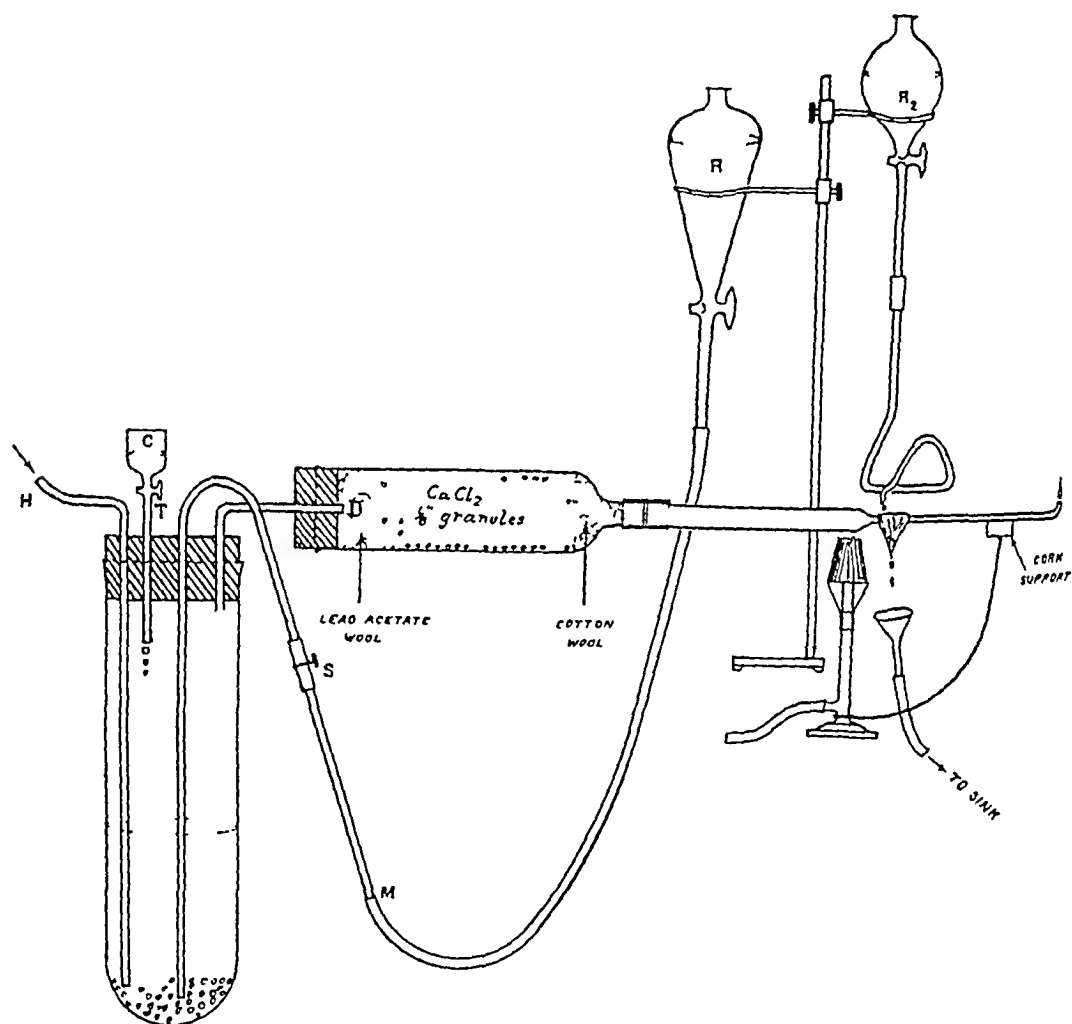
TEXT FIGURE

water out of D and connect the $CaCl_2$ tube (5 in $\times \frac{3}{4}$ in.) which is in turn connected with the mirror-tube (*see* Text-figure)   The body of the mirror-tube is about 3 inches and the capillary, 5 inches long

The reservoir (R_2) serves as a constant dropping arrangement of water, on the pad of cotton, placed $\frac{1}{2}$ inch away from the constricted neck of the mirror-tube, which is heated by a Bunsen-flame placed below   This constant water-dropping arrangement serves as a condenser for arsenic and is essential

Pass a slow current of hydrogen from the gas-holder for one minute   Light the hydrogen at the tip of the capillary tube which is kept burning during the experiment with an $\frac{1}{8}$ inch flame   Drop in HCl (50 to 70 per cent) from C and run a blank for 5 minutes to test the purity of the chemicals used, which is indicated by the absence of any stain of arsenic   The food-digest is now transferred into C and the experiment is run for 20 to 25 minutes dropping nearly 1 c c to 2 c c of the digest at a time, followed by a few c c of HCl (50 to 70 per cent) to keep up the hydrogen flame at $\frac{1}{8}$ inch during the course of the experiment   After the expiry of the requisite time, T is opened   Seal the distal end first and then the proximal end, taking care not to heat the arsenic-mirror which gets diffused if heated   These mirrors were compared with standard ones, obtained from various strength of 0 1 per cent of As_2O_3 solution, on dilution

Digestion of food-materials was carried out by the wet combustion method of Ramberg on 10 grammes of material   Ten c c of HNO_3 (Sp Gr 1 4) were added, followed by 5 c c of concentrated H_2SO_4 The mixture was allowed to stay over-night   This pre-digestion has been found by the author to make further oxidization quicker than otherwise   The final digest (5 c c), after ammonium oxalate treatment, was diluted with 5 c c water, cooled and made up to 15 c c or 20 c c with concentrated HCl

With food-stuffs containing higher percentages of arsenic an aliquot part was used for the estimation and results calculated accordingly

The following samples of Indian food-materials were analysed in duplicates and quadruplicates whenever necessary with the following results —

Sea fish (Puri)	1	Indian whitings (lady's finger)	0 050	grain	per	lb
	2	Sprats (Jhudung)	0 021	„	„	„
	3	Chilka fish	0 018	,	„	„
River fish	4	Hilsha fish	0 035	„	„	„
	5	Lobster	0 025	„	„	„
	6	Rohit fish (Gâda)	0 004	„	„	„
Goat	7	Flesh	0 004	„	„	„
	8	Liver	(i) 0 046	(infected ?)		„
			(ii) 0 005	grain	per	lb
	9	Kidney	0 019	„	„	„
Cow	10	Flesh	0 008	„	„	„
	11	Liver	0 018	„	,	„
	12	Kidney	a trace			
	13	Chicken	a trace			
	14	Mohua (flower)	0 004	„	„	„
Cereals	15	Lentils				
	16	Arhar				
	17	Mung				
	18	Wheat (atta)	} a trace			
	19	Flour (maida)				
	20	Rice (atap, washed and dried)				
Vegetables	21	Potato				
	22	Brinjal				
	23	Potôl				
	24	Jhinga				

In conclusion, I beg to thank the Director of Public Health, Bihar and Orissa, for his kind permission to carry on this work in his laboratories

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THE COCCIDIA OF LIZARDS.

BY

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AND

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IN examining the intestinal contents of geckoes in order to obtain material for students to study, we have come across three species of *Eimeria* and one of *Isospora*, which we may here describe The lizards in question were kindly identified for us by Dr Baini Prashad, D SC, F R S E, F A S B, Director, Zoological Survey of India, Calcutta, as *Hemidactylus flaviviridis* (Ruppell) Unfortunately we have seen only the sporogony cycle in all four species, and although sections of the gut and liver were cut and examined in every instance, we have not seen the schizogony cycle

Eimeria, species A – The oocyst of this species is shown in Plate **XXX**, figs 1 to 5 and in figs 15 and 16 It is almost spherical in shape Fifty oocysts were measured with the ocular micrometer and gave the following figures —

smallest	15 3μ	$\times$	13 6μ
mean	18 02 $\pm$ 1 861μ	$\times$	16 15 $\pm$ 1 819μ
largest	21 2μ	$\times$	20 4μ

In other words the oocysts ranged in size from 16 to 20 microns in length by 14 to 18 microns in breadth The infection was very abundant in one lizard and all phases up to the mature oocyst with four sporocysts, each containing two sporozoites, were observed in the gut contents The sporocysts show no cap, such as that described by Triffit (1925) in *Eimeria pythonis*, and no micropile was visible in the oocysts

EXPLANATION OF PLATE XXX

Camera-lucida drawings of the oöcysts

Figs 1—5   *Eimeria, species A*
 „   6—9   *Eimeria, species B*
 „   10—13   *Eimeria, species C*
Fig 14   *Isospora* sp

Photomicrographs of the oocysts

Figs 15 and 16   Oocysts of *Eimeria, species A*, showing sporoblastic masses
Fig 17   Oocyst of *Eimeria, species B*, with four well-differentiated sporoblasts
Figs 18 and 19   Oocysts of *Eimeria, species C*   Fig 18 shows a single mass of protoplasm   Fig 19 shows four sporoblasts lying on different planes (Lower magnification)
Fig. 20   Oocyst of *Isospora* sp , showing two typical sporoblasts

PLATE XXX

1 2 3 4 5 6 7 8

9 10 11 12 13 14

0   60 μ
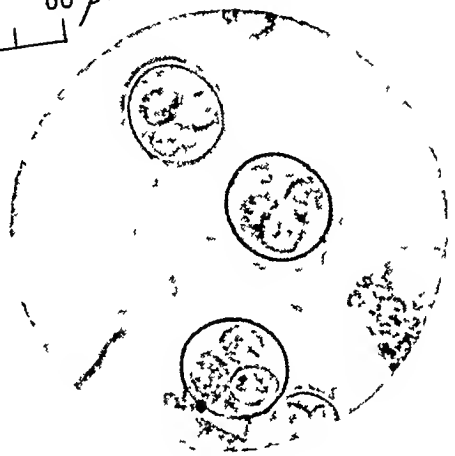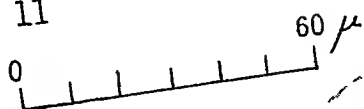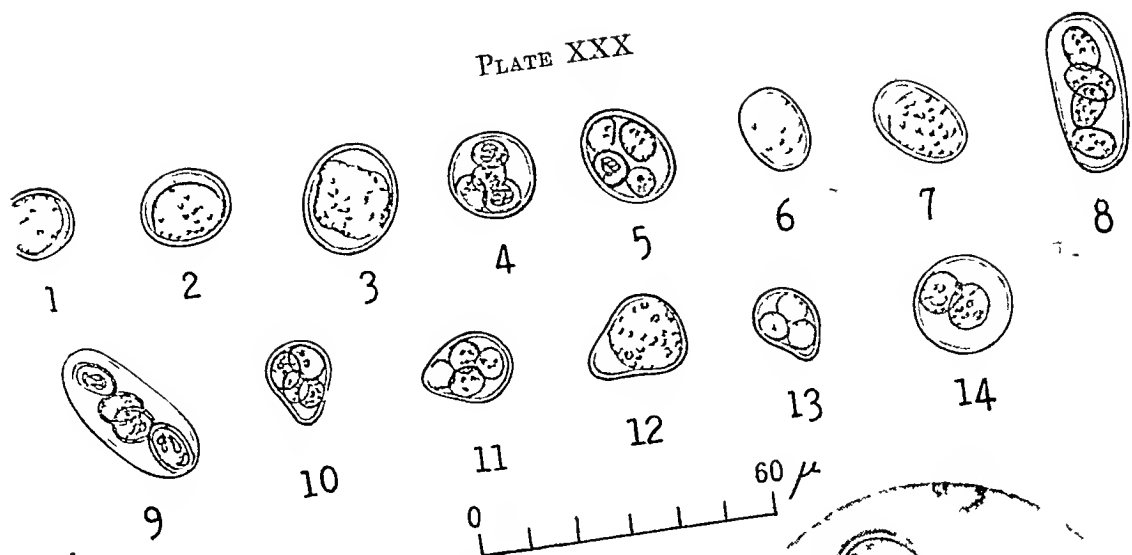
15 16
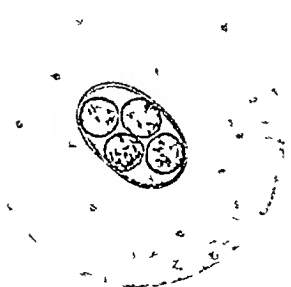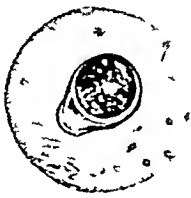
17 18 19

20

The development of the sporogony cycle was followed up in a moist chamber preparation

DISCUSSION

Very little work has been done on the coccidia of lizards, and unfortunately several of the papers published on the subject date back to the 19th century and are not available anywhere in India Under these circumstances we are very deeply indebted to Captain R L Sheppard, Secretary of the Bureau of Hygiene and Tropical Diseases, London , to Miss Bellis, Librarian of the Wellcome Bureau of Scientific Research , and to Dr Cecil Hoare of the same Bureau, for looking out this literature for us and for detailed abstracts from it A summary of the previous work on this subject is given in the Table

With regard to the genus *Eimeria*, Wenyon (1926) states that Eimer (1870) described a species of *Eimeria* from lizards This, however, appears to be a mistake Eimer's paper is based on the finding of organisms in three mice, and there is no mention of any lizard The mistake appears to have originated with Labbé (1899) whose 'Sporozoa' in *Das Tierreich* one may call the bible of the 'sporozoologist' Here, on p 70, he gives a reference to a species of *Lacerta* in connection with Eimer's work, whereas Eimer himself gives a list of small animals other than mice in which coccidia had been found, though he makes no mention of a lizard From Labbé (*loc cit*) the mistake appears to have been copied into the later literature

Wenyon (*loc cit*) also states that Danilewsky in 1896 described an *Eimeria* from a species of *Lacerta*, but Danilewsky is credited by Stiles and Hassall (*Index-Catalogue of Medical and Veterinary Zoology*) with only one paper in 1896, and that deals with malaria A paper by Danilewsky (1886) was finally run to earth in the British Museum, and we have received a detailed abstract from the original from the Bureau of Hygiene and Tropical Diseases This abstract was shown to Dr Wenyon before it was sent to Calcutta, and he gives it as his opinion that the cysts from the kidney and liver referred to by Danilewsky are merely the mature schizonts of the hæmogregarines which he was studying in the blood of some lizards On reading very carefully through the abstract we agree entirely with Dr. Wenyon Danilewsky everywhere compares the structures which he is describing with the gregarines, but he makes no mention of a coccidium at all

Leger, L (1898), described an *Eimeria—E raillieti—*from the lizard *Anguis fragilis* with oocysts 18μ in diameter , but we have been unable to secure this paper in the original in India, and so far it has not been unearthed in London

Laveran and Petit (1910) described an *Eimeria—E agamæ—*from the lizard *Agama colonorum* This paper we have been able to consult in the original Schizogony occurs in the ramifications of the bile ducts in the liver, the cylindrical epithelial cells being infected and their nuclei flattened into a cruciform shape by the growing parasites The oocysts are oval, from 20μ to 25μ in length by 11μ to 14μ in breadth The sporocysts are fusiform, and measure 8μ by 4μ Free sporozoites were also seen in fresh preparations

Phisalix (1923) has described an *Eimeria—E scinci—*from the lizard *Scincus officinalis* This paper we have also seen in the original The schizogony cycle

occurs in the biliary ducts in the liver and in the gall-bladder, in the case of adult lizards only the sporogony cycle is seen, the schizogony cycle having apparently died out   (This may explain our want of success in seeking for the schizogony cycle)   The mature oocysts are ellipsoidal, measuring 36μ by 25μ [*not* 32μ by 25μ as stated by Triffit (*loc cit*)   This is probably a misprint which has been copied in Wenyon's ' Protozoology ']

Recently Setna (*loc cit*) has described an *Eimeria* in the gall-bladder of *Hemidactylus flaviviridis*   He states that all stages of development occur in the columnar cells of the gall-bladder, the mature oocysts are oval, measuring 24μ to 34μ in length by 11μ to 14μ in breadth   The sporocysts measure 9μ by 7μ He does not name the parasite, or discuss the literature   The dimensions of this parasite are larger than those of our ' species B', but they appear to correspond with the occasional giant forms of the latter

The above information is admittedly not complete, but it is all that is available   If we turn to the three species of *Eimeria* which we have described above in *Hemidactylus flaviviridis*, ' species A' may correspond to *Eimeria railliei* Leger, 1899, but in the absence of detailed information it is impossible to make certain   ' Species B' appears to correspond fairly well to *Eimeria agamæ* Laveran and Petit, 1910 Unfortunately we have not been able to see the schizogony cycle of ' species B', so cannot make certain on this point

' Species C' appears to be new   The curious lemon-like shape of the oocysts and the fact that many of them are bile-stained clearly differentiate it from the species already described   For this parasite we suggest the name *Eimeria hemidactyli* nov sp

With regard to the genus *Isospora*, Hagenmuller (1898) has described a species —*I camilleri*—from the lizard *Gongylus ocellatus* (now *Chalcides bedrigæ*)   The oocysts (called ' sporokystes ' by the author) measure 22μ in diameter, are spherical and with a double contoured wall   Each, when fully developed, contains two oval sporocysts (called by the author ' spores ') with dissimilar poles, without any residual body   One pole of the sporocyst is drawn out into a very short ' neck ' with a thickening at the end, the opposite pole is rounded   The sporocyst measures 18μ $\times$ 12μ, contains four sporozoites arranged spirally, and a sporocystic residue, which is represented by a few granules and is sometimes absent   The author names this parasite *Diplospora camilleri*, but it should of course be *Isospora camilleri*   There are no illustrations to his article

Sergent, Ed (1902), has described *I mesnili* from *Camæleon vulgaris*, this parasitizes the nuclei of the intestinal epithelium, whilst the oocysts were about 30μ in length, and the sporocysts 16μ by 10μ Wenyon (1926, **2**, p 826) mentions having found an *Isospora* in the liver and intestine of *Agama colonorum* in the Sudan, but gives no further details   He also states that Adler had found an *Isospora* in the lizard *Varanus griseus* in Palestine, this develops in the sub-epithelial tissues of the small intestine

The *Isospora* which we have found in *Hemidactylus flaviviridis* appears to have a smaller oocyst than *Isospora mesnili* Sergent, 1902, but the amount of material at our disposal was so scanty that we prefer not to give a specific name to the parasite which we have encountered

TABLE

Coccidia of lizards previously described

Author	Host	Name of parasite	Size of oocysts	Remarks
		Genus *Eimeria*		
Leger (1899)	*Anguis fragilis*	*E. ranhei*	18µ in diameter	.
Laveran and Petit (1910)	*Agama colonorum*	*E. agamæ*	Oval 20 to 25µ × 11 to 14µ	Schizogony in liver and bile ducts
Phisalix (1923)	*Scincus officinalis*	*E. scinci*	Ellipsoidal 36µ × 25µ	Schizogony in liver and gall-bladder
Setna (1933)	*Hemidactylus flaviviridis*		24 to 34µ × 11 to 14µ	All stages in the gall-bladder
		Genus *Isospora*		
Hagenmuller (1898)	*Gongylus ocellatus*	*I. camilleri*		
Sergent (1902)	*Cameleon vulgaris*	*I. mesnili*	30µ long	Schizogony in gut epithelium
Wenyon (1926)	*Agama colonorum*			Schizogony in liver and gut Found in the Sudan
Adler, quoted by Wenyon (1926)	*Varanus griseus*			Schizogony in epithelium of small intestine Found in Palestine

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*Not seen in the original

AN ENTAMŒBA OF THE GECKO

BY

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WHILST examining the intestinal contents of geckoes in order to obtain material for students to study, we have come across an interesting entamœba in *Hemidactylus flaviviridis* This infection is not very common, as it was seen in only two out of about thirty lizards examined

Cultures were taken in the media used by Barret and Smith, Cleveland and Sanders, and Dobell and Laidlaw's HSre + S medium, and kept at room temperature In these the organism multiplies very slowly, but a fair growth is obtained about two weeks after inoculating the media

In the motile trophozoite phase, the entamœba is actively motile, and closely resembles *Entamœba histolytica* of man It changes its locality rapidly and soon travels out of the microscope field About one-third of the animal consists of clear ectoplasm The direction of movement is not more or less in a straight line as with *E histolytica* but variable, the pseudopodia being pushed out first in one direction, then in another, then in a third There is a flowing movement of the whole animal in locomotion rather than the extrusion of ectoplasm only The amœba is a voracious feeder, ingesting bacteria and the like, and in culture it feeds avidly on starch grains

The nucleus very closely resembles that of *E histolytica*, and is invisible in the fresh preparation in saline In iodine and in films fixed by Schaudinn's fixative and stained by iron-hæmatoxylin, it is seen that there is a very thin uniform deposit of 'peripheral' chromatin on the inner aspect of the nuclear membrane, and a fine central karyosome, the space between the peripheral chromatin and the karyosome being free from chromatin The nucleus is thus typical of the genus *Entamœba*

EXPLANATION OF PLATE XXXI

Camera-lucida drawings of the entamœba in 1 per cent iodine solution

Figs 1—10   Trophozoites, some with ingested starch granules

,, 11 and 12   Trophozoites undergoing division

,, 13—18   Cysts with varying number of nuclei   Fig 18 shows a cyst with ten nuclei

Photomicrographs of the entamœba in culture in 1 per cent iodine solution

Fig 19   Trophozoites with nucleus showing a central karyosome   The amœbæ have ingested starch granules from the medium

(Leitz Obj $\frac{1}{6}$, Oc 5 ×)

,, 20   Cyst with six nuclei lying on different planes

(Leitz Obj $\frac{1}{6}$, Oc 5 ×)

Figs 21 and 22   Trophozoite and cyst respectively

(Leitz Obj $\frac{1}{6}$, Oc 10 ×)
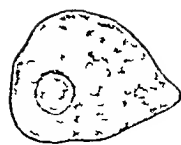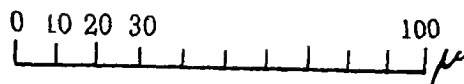
PLATE XXXI

Fifty-six individuals in the trophozoite phase which had more or less rounded up were measured with the ocular micrometer, and gave the following results —

<div style="text-align:center">

smallest 28 0μ
mean   32 2μ
largest   38 4μ

</div>

Plate XXXI, figs 1 to 12, shows the appearance of the trophozoite phase as seen in 1 per cent iodine emulsion, drawn with the camera lucida   The ingestion of starch grains is well seen in Figs 5 to 12, whilst Fig 11 shows an individual undergoing division   Figs 19 and 21, Plate XXXI, also illustrate the motile, trophozoite phase

Occasionally the organism encysts in Dobell and Laidlaw's medium, whilst cysts were also present in the lizards' gut contents   These are illustrated in Figs 13 to 18 and in Figs 20 and 22 of Plate XXXI   The cyst closely resembles that of *Entamœba coli* of man, and has the same ' beaded ', almost granular appearance—especially when seen in the iodine preparation   The early cysts possess a large mass of glycogen, which occupies almost the whole of the cyst, but as the cyst matures this is used up   The mature cyst usually has 8 nuclei, but more than 8 are not infrequently present   Forty-two cysts were measured with the ocular micrometer and gave the following dimensions —

<div style="text-align:center">

smallest 16 8μ
mean   17 8μ
largest   18 4μ

</div>

The nuclei in the cysts remain true to type, with a very thin deposit of peripheral chromatin on the inner aspect of the nuclear membrane and a fine and delicate karyosome   The karyosome is almost always central in position within the nucleus, but occasionally it is placed a little eccentrically

DISCUSSION

Very few entamœbæ of lizards appear to have been described   *Amœba lacertæ* Hartmann, 1907, was so named by Hartmann and Prowazek (1907), and studied by Nagler (1909)   Dobell (1914) gives a very detailed account of this organism, which he states to be a fairly common inhabitant of *Lacerta muralis*   His description of the nuclear characters and of the cyst, which is mononucleate, and his plate show that this organism is quite a different one from that which we have just described   On the other hand, in the same paper, Dobell describes a second organism—an *Entamœba*—from *Lacerta muralis*   Small individuals when rounded up measure 10μ or less, and large ones 30μ or more   The nucleus contains a very large karyosome, surrounded by numerous granules of peripheral chromatin supported on a linin net-work   Dobell does not name the organism, or describe the cyst   He notes that when alive it has an appearance (the nucleus excepted) very like that of *Entamœba ranarum*, and it moves in a similar manner

Our organism may be the same as Dobell's entamœba in *Lacerta muralis*, but the nuclear characters of the two appear to differ   In all the specimens which we have seen the karyosome is always very small, delicate and almost always central in position

Wenyon and O'Connor (1917) mention an entamœba in *Agama* sp , which was found in both the motile and encysted phases   They note that it resembled *E coli* of man and produced an 8-nucleate cyst indistinguishable from that of *E coli* Later, Wenyon (1920) described what is apparently the same entamœba from *Lacerta agilis* and *Agama stellio*   He notes that it bears a striking resemblance to *E coli,* and feeds upon the intestinal contents of the lizard, frequently ingesting intestinal flagellate protozoa and their cysts   It produces an 8-nucleate cyst which is indistinguishable from that of *E coli*   His plate of the trophozoite forms shows only scanty ectoplasm, and a large karyosome in an eccentric position   Whether our organism is the same as Wenyon's or not, we find it difficult to say   The nuclear characters appear to be different, and in its motile phase it bears a close resemblance to *E histolytica,* and not to *E coli*

Franchini (1921) describes an entamœba from *Chalcides ocellatus*   It varied very much in size, the cysts were almost always 4-nucleate, very rarely 8-nucleate No illustration is given, and no measurements, and it would be very difficult for any worker to identify this entamœba

The entamœba in *Hemidactylus flaviviridis* is certainly not *Amœba lacertæ* It appears to differ from the entamœba of *Lacerta muralis* described by Dobell Wenyon's organism in *Lacerta agilis* and *Agama stellio* appears again to be a different species, and in any case Wenyon did not name it   The cysts of the entamœba of *Hemidactylus flaviviridis* appear to be entirely different from those of Franchini's organism , and also the latter was not fully described or named   We suggest, therefore, for the entamœba which we have described, the name *Entamœba flaviviridis* sp   nov

We are much indebted to Mr H M Roy, artist of the Calcutta School of Tropical Medicine, for the camera-lucida drawings (Figs 1 to 18) in Plate XXXI

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CARRIERS OF *V. CHOLERÆ* WHO ENTER CEYLON FROM SOUTH INDIA

BY

LUCIUS NICHOLLS, M D   B C , B A (Cantab)

(*From the Bacteriological Institute, Colombo*)

INTRODUCTION

LARGE areas in Ceylon have been planted in tea and rubber during recent years, much labour was required for these cultivations , the greater part of this has been supplied by the poorest classes of the Madras Presidency , and thus a large migration has taken place from Southern India to Ceylon

The Indian population on the estates of Ceylon numbered 740,130 in 1929 , it has fallen recently owing to the depressed times when some estates have been closed down, and labourers have been repatriated

In addition to the estate labourers many other 3rd class passengers travel yearly , and the average annual number of all 3rd class travellers to Ceylon during the decade 1924 to 1933 was —

Estate labourers	. 102,305
Other 3rd class passengers	109,825
Total	212,130

There are four areas in the Madras Presidency from which estate labourers are recruited and these have been named for statistical purposes the Trichinopoly area, the Vellore area, the Salem area and the Madura area   These areas are indicated approximately in the sketch map (Map 1)

Records have been kept of the actual districts from which estate labourers and other 3rd class passengers have come   The relative district contributions to this migration to Ceylon are shown in Graph 1

The migration takes place over two routes, namely, via Dhanushkodi and Tuticorin (*vide* Map 1)   About two-thirds of all 3rd class passengers travel by the former route

A narrow strip of land stands out from Southern India to end at Dhanushkodi and a similar strip projects from Ceylon to end at Talaimannar   These two extreme points are separated from one another by 25 miles of shallow sea   The Ceylon Government built a railway to Talaimannar, where it ends upon a pier , the South Indian Railway Company did likewise to Dhanushkodi, and also ran ferry steamers

between the two piers, the route thus established was opened in the year 1914 The Government of Ceylon built a temporary quarantine camp near Mandapam and replaced this in 1918 by a model camp in the same neighbourhood (*vide* Map 1)

Mandapam camp is situated 23 miles from Dhanushkodi and is well appointed for the comfort of all classes of passengers, the buildings have verandahs and are

MAP 1

SOUTH INDIA

VELLORE . Madras
AREA

SALEM AREA

TRICHINOPOLY AREA

MADURA AREA

Mandapam

Dhanushkodi

Tuticorin

Talaimannar

CEYLON

Colombo
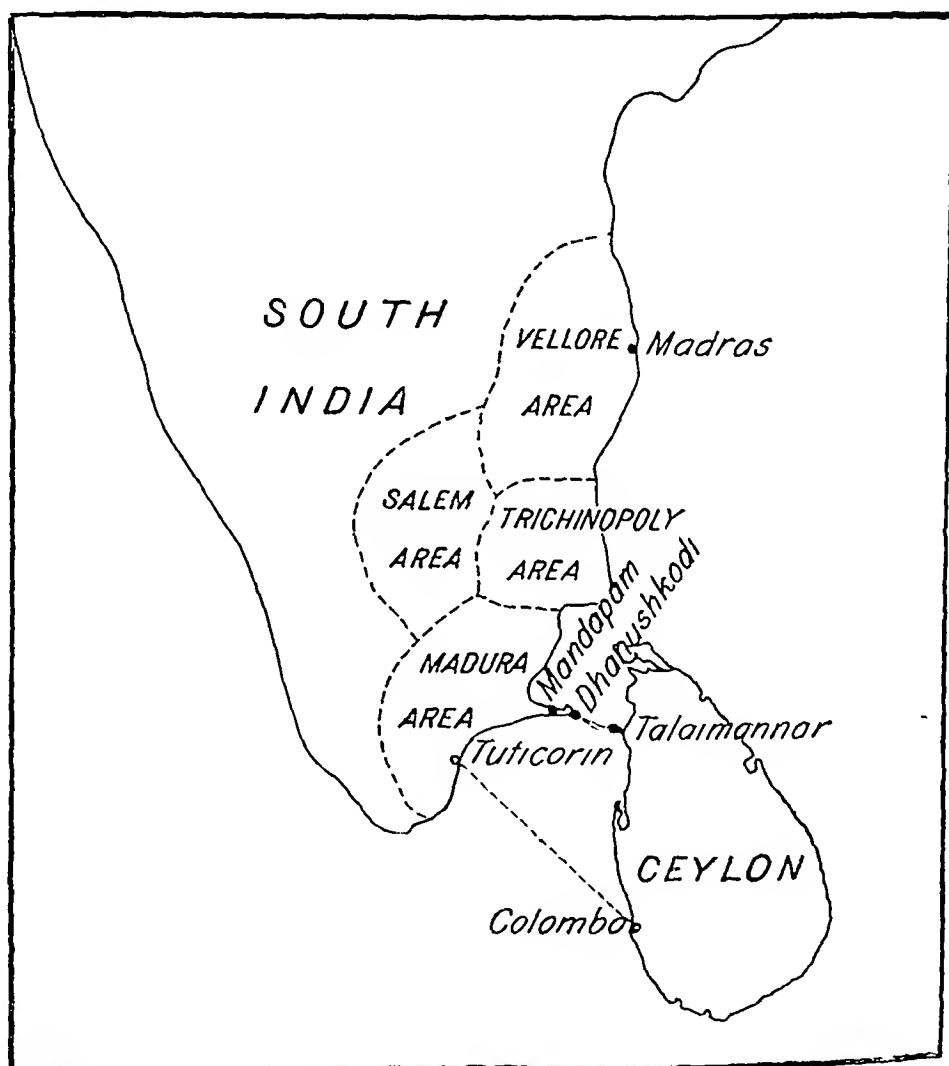
of brick, with cement floors and tiled roofs, pipe lines convey water to all the buildings and there is a water carriage sewage service and electric light throughout the camp

The trains of the South Indian Railway stop at the camp station, the passengers are medically inspected, and all estate labourers, and many other 3rd class passengers, are detained and quarantined for 5 days, not including the days of

arrival or departure   (There is another quarantine camp near Tuticorin for those
who travel by that route)

The estate labourers are vaccinated and treated for hookworm infection   The
rest and the good food produce a marked improvement in the appearance of many
of them

A small laboratory was built in 1930 and, on instructions from Dr Brierchffe
(Director of Medical and Sanitary Services of Ceylon), it was equipped for the

GRAPH 1
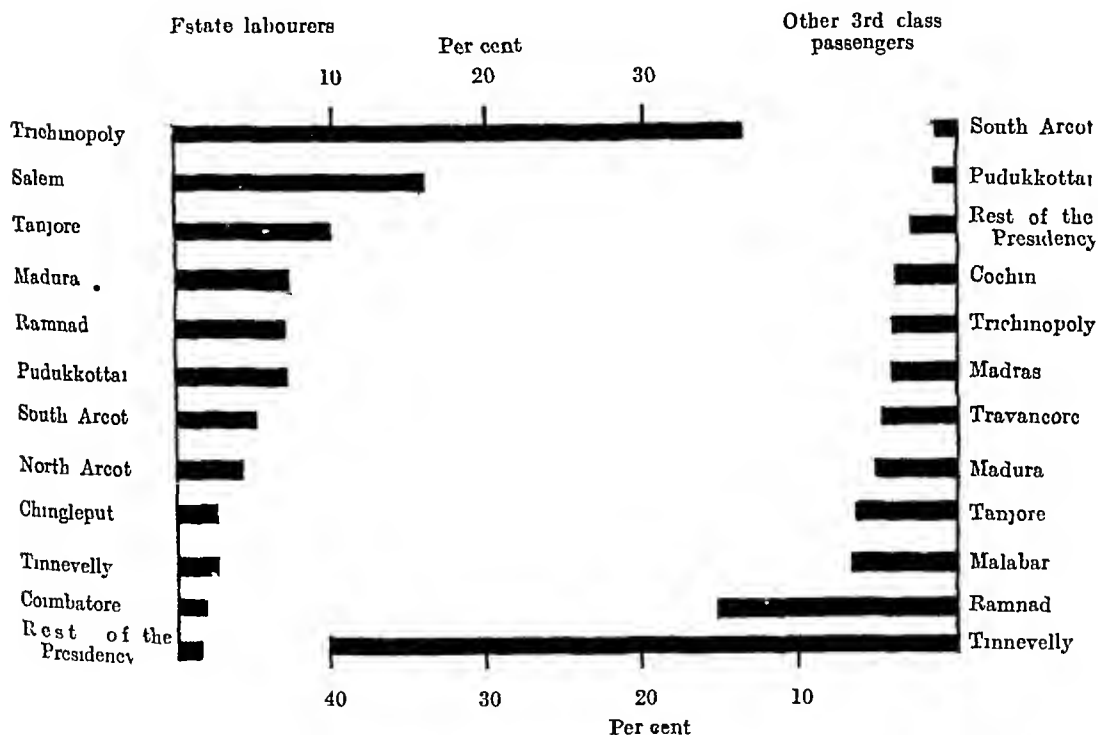
Showing districts in South India from which the estate labourers and other 3rd class passengers came to
Ceylon for the decade 1921 to 1930 (*Census of India*, Volume XIV, 1931)

examination of the stools of as many labourers and other passengers as possible for
the presence of *Vibrio cholerœ*   The investigation was started in the latter part
of 1930 and has continued since that time *

It was decided that any one who was found to harbour agglutinable *V cholerœ*
should not be detained but allowed to continue the journey to Ceylon

Table I shows the number of estate labourers and others who were quarantined
at Mandapam camp from 1st January, 1931, to July 1934

* The laboratory work at Mandapam was carried out by K M M Michael, Laboratory Assistant,
whose excellent work I have to acknowledge (L N)

TABLE I

Return showing the number of estate labourers and others who have passed through Mandapam camp monthly during the years from January 1931 to July 1934

Months	1931		1932		1933		1934	
	Labourers	Passengers	Labourers	Passengers	Labourers	Passengers	Labourers	Passengers
January	1,654	4,834	1,870	3,906	911	3,159	2,890	3,742
February	2,099	3,383	2,663	3,239	1,070	3,241	4,873	3,926
March	4,054	5,460	3,119	4,593	1,652	4,022	8,580	3,902
April	4,799	4,823	4,318	3,860	1,364	3,766	8,534	3,900
May	8,717	4,912	6,275	4,446	1,604	4,029	20,712	4,299
June	11,891	4,423	8,275	4,276	2,399	3,154	28,537	3,902
July	10,678	4,671	7,120	4,276	2,811	4,043	22,410	4,845
August	4,876	3,358	4,314	3,024	2,289	3,098		.
September	6,558	4,497	5,287	4,082	3,363	4,016		
October	5,613	3,672	3,527	3,498	4,020	3,263		
November	3,391	3,608	2,176	3,109	5,789	3,159		.
December	3,407	2,833	1,925	3,663	5,620	3,518		
TOTALS	68,337	50,474	50,869	45,972	32,898	42,408	96,545	28,506

Cultural methods

Collection of samples —Small tins and sticks were issued to the estate labourers and other 3rd class passengers for the collection of samples of their stools on the *3rd day of their stay in camp*

Examination of samples —It was considered desirable to examine a very large number of samples, and, therefore, ten samples (occasionally fewer) were thoroughly mixed and a portion of the mixture was placed in a 100 c c Erlenmeyer flask containing 35 c c of peptone water   On each day a number of single samples were also examined   This method was continued from 1st January, 1931, to February 1933, when it became apparent that the examination of mixed samples was not as satisfactory as the examination of single samples , therefore they were discontinued, and after February 1933 all samples were examined singly

The medium used was 1 per cent peptone 0 5 per cent sodium chloride in tap water adjusted to pH 8   This was distributed into 100 c c flasks for mixed samples and into 5″ × 5″ test-tubes for single samples   The media in the flasks and tubes were inoculated with 2 loopfuls of the samples (approximately 0 1 gramme) and after 6 hours' incubation at 37°C loopfuls were taken from the surface of the media, and sub-cultured into large test-tubes of peptone water   The next morning these sub-cultures were examined for motile vibrios, when these were present nutrient agar plates were seeded and 24 hours later colonies of vibrios were examined for agglutination by the microscopic method

Sub-cultures were made from each plate on which agglutinable vibrios grew and these were further studied at the Mandapam camp laboratory and at the Bacteriological Institute in Colombo

Also sub-cultures were made from a large number of colonies of non-agglutinable vibrios for subsequent examination

Agglutination tests

All cultures which had shown agglutination by the hanging drop microscopical method, and many cultures, which had not shown this agglutination, were examined by the macroscopical standard agglutination method   Two sera were used simultaneously in most of the tests —

(1) A serum, here designated Berne serum, which had been obtained several years ago from Berne, and is in the form of a dry powder in ampoules, and has been kept in cold storage and has retained a fairly high titre for *V choleræ*

(2) A serum, here designated Wellcome, which was used soon after it was received from the Wellcome Physiological Research Laboratories

The organisms to be tested were seeded into measured quantities of peptone water and incubated at 37°C for 24 hours   Formalin was added so that the cultures contained 0 2 per cent, and the cultures were diluted with less than equal quantities of 0 2 per cent formalin in normal saline so as to bring them to the same opacity by comparison with an arbitrarily selected standard opacity tube

The formalized cultures were kept in a cold storage machine for one or two days before being tested for agglutinating titre

Serial dilutions of the sera were made, a quantity of the diluted serum was mixed with an equal quantity of the standardized suspension for each dilution to be tested. The dilutions made ranged from 1 in 50 to 1 in 10,000. The tubes were incubated for two hours on a water-bath maintained at a temperature of 55°C (approximately). When the tubes were taken from the water-bath they were kept at room temperature for 15 minutes before the reading was made. The usual controls were made for each suspension tested.

The highest dilution in which there was marked agglutination was accepted as the end-point of the titre, agglutinations of lesser degree were ignored.

Table II gives the agglutinating titre for the majority of the agglutinating vibrios which were isolated from 'carriers', it shows that the titre varied from 1 in 100 to 1 in 10,000.

The vibrios which have been isolated are placed into three groups.

Group I vibrios which were agglutinated by the standard agglutination method in dilutions of not less than 1 in 100 with Berne or Wellcome sera. These vibrios all showed high motility, were typical morphologically of *V choleræ*, gave the nitroso-indol reaction and fermented saccharose, dextrin, glucose, maltose, galactose, mannose and lævulose, with the formation of acid only. They did not ferment xylose, dulcitol, isodulcitol, adonitol or inositol. A few of them showed some production of acid in peptone water containing lactose.

Group II vibrios which were non-agglutinable with 'cholera' serum, but were morphologically indistinguishable from *V choleræ*, gave the nitroso-indol reactions and the same fermentation tests as vibrios of group I.

Colonies of these vibrios were a little more opaque than most colonies of the agglutinating vibrios.

There were occasions on which plates, seeded for the isolation of vibrios, showed colonies with sectors, segments or narrow lines slightly more translucent than the remaining areas of the colonies. Agglutinable vibrios of group I were cultured from these translucent areas but only non-agglutinable vibrios of group II could be cultured from the more opaque areas. Cultures have been obtained which continued for months to give rise to those mixed colonies whenever plates were seeded from them.

It appears that *these colonies contain two variants of the same species, and are not a mixture of two species*, because on a number of occasions only 'mixed' colonies and colonies of non-agglutinable vibrios have grown on seeded plates, pure colonies of agglutinable vibrios being absent.

If it is assumed that two species are growing in those 'mixed' colonies, then it is necessary also to assume that these two species have such an astonishing attraction for one another that they fall together on the same points on the media in the plates, because in many plates the area of the media which showed no growth was far greater than the area covered by mixed colonies, or colonies of non-agglutinable vibrios. This subject will be discussed later in this paper.

Group III vibrios which were non-agglutinable with cholera serum and differed morphologically from *V choleræ* did not give the nitroso-indol reaction, and the few which were tested did not ferment saccharose. Records

were kept of the number of occasions on which these organisms were isolated, but they did not receive the amount of detailed attention which was given to the organisms of groups I and II

LYSIS BY BACTERIOPHAGE

All the agglutinable vibrios and a few of the non-agglutinable vibrios were tested for lysis with a preparation of 'phage obtained from the Medical Research Institute, Shillong   The preparation contains three strains of 'phage, each of which lyses a different strain of *V choleræ*

Two tubes of peptone water were inoculated from each culture to be tested, 'phage was added to one and this was compared for lysis with the other which had not been inoculated with the 'phage   The results were read after 24 hours' incubation at 37°C and were recorded with plus or minus signs, $+$ signified some lysis, and $+++++$ signified complete lysis

Time did not permit of a detailed study of the effect of different strains of 'phages upon the vibrios isolated, but the results are sufficient to show that the lytic effect of the 'phage used varied from complete absence of lysis in some strains to complete lysis in others   Furthermore, the agglutinable strains as a group could not be distinguished from the non-agglutinable strains by the action of the bacteriophage used (*vide* Table II)

TABLE II

Number of culture	Month of isolation	Highest titre of agglutination with Berne serum	Highest titre with Wellcome serum	Degree of lysis by Shillong 'phage	Number of culture	Month of isolation	Highest titre of agglutination with Berne serum	Highest titre with Wellcome serum	Degree of lysis by Shillong 'phage
	1931				8	June	N T	2,000	++
1	May	N T	800	+	9	,,	N T	2,000	++
2	,,	3,200	1,600	+	10	Dec	N T	6,400	+++
3	,,	200	200	+++		1932			
4	June	N T	2,000	+++	11	Jan	2,000	8,000	++
5	,	N T	6,400	++++	12	,,	1,600	4,000	+++
6	,	200	200	+	13	,,	2,000	8,000	++
7	,	N T	2 000	++	14	,,	3,200	10,000	++

N T = Not tested

TABLE II—*contd*

Number of culture	Month of isolation	Highest titre of agglutination with Berne serum	Highest titre with Wellcome serum	Degree of lysis by Shillong 'phage	Number of culture	Month of isolation	Highest titre of agglutination with Berne serum	Highest titre with Wellcome serum	Degree of lysis by Shillong 'phage
15	Jan	3,200	10,000	+++	38	Oct	800	800	—
16	,,	3,200	10,000	+	39	,,	1,600	1,000	—
17	,,	3,200	10,000	+++		1933			
18	Feb	2,000	800	++	40	Jan	2,000	1,600	+
19	April	800	200	+++++	41	Feb	200	800	+
20	,,	4,000	8,000	+++	42	,,	2,000	2,000	++
21	,,	6,400	6,400	++	43	.,	1,600	1,000	++
22	June	2,000	10,000	+	44	April	2,000	800	—
23	,,	4,000	1,000	+++	45	May	3,200	4,000	++++
24	,,	3,200	2,000	++	46	,,	200	· 100	+++++
25	,,	2,000	1,000	++	47	Sept	3,200	2,000	+
26	,,	1,000	1,600	++++	48	,,	4,000	3,200	++
27	,,	1,600	10,000	++	49	,,	1,600	800	++
28	,,	3,200	1,600	++	50	,,	3,200	1,600	+++
29	,,	3,200	10,000	++++	51	,,	2,000	1,000	++
30	,,	2,000	4,000	—	52	,,	3,200	1,600	+
31	,,	2 000	3,200	++	53	,,	2,000	1,600	++
32	,,	3,200	3,200	+	54	Oct	4,000	1,600	+
33	,,	2,000	¯1,000	—	55	,	3,200	1,000	++
34	,,	2,000	1,600	—	56	,,	3,200	1,600	++
35	,,	1,600	1,000	+	57	,,	50	100	—
36	Sept	4,000	6,400	+	58	,,	2,000	1,000	++
37	Oct	800	800	—	59	Nov	1,600	1,000	—

TABLE II—*concld*

Number of culture	Month of isolation	Highest titre of agglutination with Berne serum	Highest titre with Wellcome serum	Degree of lysis by Shillong 'phage	Number of culture	Month of isolation	Highest titre of agglutination with Berne serum	Highest titre with Wellcome serum	Degree of lysis by Shillong 'phage
60	Nov	3,200	1,000	++	72	June	2,000	3,200	+++
61	Dec	2,000	1,000	N T	73	,,	2,000	4,000	+
							Non agglutinable vibrios		
	1934					1932			
62	Jan	2,000	1,600	+		Feb	—	—	+
63	Feb	400	200	+++		Dec	—	—	++++
64	March	400	200	+++		1933			
65	April	50	100	+++		Jan	—	—	—
66	,,	50	400	+		April	—	—	+++++
67	,,	50	200	++		,,	—	—	+++++
68	,,	1,600	1,000	—		July	—	—	—
69	May	1,600	1,000	—		1934			
70	,,	1,600	3,200	++		Feb	—	—	+++
71	,,	3,200	8,000	+++++		,,	—	—	++++

N T = Not tested

STATISTICAL RESULTS

Samples of stools from 100,896 persons were examined between the 1st January, 1931, and 31st July, 1934   Recruited estate labourers supplied 85,558 samples and passengers supplied 15,238 samples

Agglutinable vibrios were isolated on 84 occasions , 81 of these were from estate labourers and 3 from passengers   Non-agglutinable vibrios as defined for group II were isolated on 2,838 occasions, and of these 2,710 were from estate labourers and 128 from passengers

Non-agglutinable vibrios as defined for group III were isolated on 992 occasions and of these 931 were from estate labourers and 61 from passengers

The results for each year are given in Tables III to IX, which are again summarized in Table X

TABLE III

1931 *estate labourers*

	Jan	Feb	March	April	May	June	July	Aug	Sept	Oct	Nov	Dec	TOTALS.
Number of persons supplying samples	829	1,584	1,734	1,298	3,240	5,480	4,957	4,747	1,592	3,912	2,802	2,530	34,705
Number of mixed samples examined	72	139	178	119	305	553	505	476	168	400	271	254	3,440 from 33,714 persons
Number of mixed samples + for *V choleræ* (group I)				·	3	5							8 ⎫ 9
Number of mixed samples + for *V choleræ* and vibrios (group II)						1							1 ⎭
Number of mixed samples + for non agglutinable vibrios (group II)	27	71	46	39	95	111	86	76	17	75	50	116	809 ⎫ 820
Number of mixed samples + for non agglutinable vibrios (groups II and III)	4	4	1		1								10 ⎫ 179
Number of mixed samples + for non agglutinable vibrios (group III)	9	20	23	12	24	42	3	5		19	5	7	169 ⎭
Number of single samples examined	114	163	146	109	168	43		37	4		106	101	991
Number of single samples + for *V choleræ* (group I)					·								·
Number of single samples + for *V choleræ* and vibrios (group II)	11	25	11	12	8	4		4			5	7	87 ⎫ 102
Number of single samples + for non agglutinable vibrios (group II)	2	7	3	2	1			1					15 ⎭
Number of single samples + for non agglutinable vibrios (groups II and III)									·				15 ⎫ 68
Number of single samples + for non agglutinable vibrios (group III)	9	7	16	4	6	2					2	6	53 ⎭

TABLE IV

1931 passengers.

	July	Aug	Sept	Oct	Nov	Dec	TOTALS
Number of single samples examined	387	875	192	684	434	591	3,163
Number of single samples + for V cholere (group I)						1	1
Do + for V cholere and vibrios (group II)	5	3		1	4	17	30
Do + for non agglutinable vibrios (group II)							
Do + for non agglutinable vibrios (groups II and III)							
Do + for non agglutinable vibrios (group III)	1	1		6	3	3	14

TABLE V.

1932 estate labourers

	Jan	Feb	March	April	May	June	July	Aug	Sept	Oct	Nov	Dec	Totals
Number of persons supplying samples	1,475	2,223	1,984	3,183	1,483	5,349	2,741	2,739	823	1,382	1,540	1,406	26,328
Number of mixed samples examined	133	221	196	313	142	516	262	259	78	125	138	119	2,502 from 24,162 persons
Number of mixed samples + for *V. choleræ* (group I)				1		11			1	1			14 }
Number of mixed samples + for *V. choleræ* (group I) and vibrios (group II)	7		1	1		3							12 } 26
Number of mixed samples + for non agglutinable vibrios (group II)	75	112	113	131	11	46	20	24	8	28	40	61	669 · 707
Number of mixed samples + for non agglutinable vibrios (groups II and III)	5	5	3	9						1	4	1	26 }
Number of mixed samples + for non agglutinable vibrios (group III)	3	13	13	23	21	12	11	6	1	3	6	1	113 } 139
Number of single samples examined	327	312	127	112	52	221	145	194	52	201	190	233	2,166
Number of single samples + for *V. choleræ* (group I)		1								2			3 }
Number of single samples + for *V. choleræ* (group I) and vibrios (group II)						1							1 } 4
Number of single samples + for non agglutinable vibrios (group II)	40	29	11	11	2			3			4	16	117 · 132
Number of single samples + for non agglutinable vibrios (groups II and III)	8										1		14 }
Number of single samples + for non agglutinable vibrios (group III)	9	7	8	2	2		3	1		4	5	2	43 } 57

TABLE VI

1932 passengers

	Jan	Feb	March	April	May	June	July	Aug	Sept	Oct	Nov	Dec	Totals
Number of persons supplying samples	634	783	700	592	225	886	428	411	112	282	471	439	5,967
Number of mixed samples examined		16	51	50	22	91	44	43	12	29	47	43	448 from 4,278 persons
Number of mixed samples + for *V. cholerae* (group I)													
Number of mixed samples + for *V. cholerae* (group I) and vibrios (group II)			7	1		3					2	8	21 } 26
Number of mixed samples + for non agglutinable vibrios (group II)								2					
Number of mixed samples + for non agglutinable vibrios (groups II and III)				1	1					1			5 } 5
Number of mixed samples + for non agglutinable vibrios (group III)													
Number of single samples examined	634	634	190	97	14	12	10	14	4	15	28	37	1,689
Number of single samples + for *V. cholerae* (group I)	1												1
Number of single samples + for *V. cholerae* (group I) and vibrios (group II)													
Number of single samples + for non agglutinable vibrios (group II)	12	4	1										19 } 21
Number of single samples + for non agglutinable vibrios (groups II and III)	2												2 } 11
Number of single samples + for non agglutinable vibrios (group III)	2	4	1							1	1	1	9

TABLE VII

1933 estate labourers

	Jan	Feb	March	April	May	June	July	Aug	Sept	Oct	Nov	Dec	TOTALS
Number of persons supplying samples	668	703	726	898	1,077	1,646	1,478	1,312	2,105	1,234	2,269	1,794	16,000
Number of mixed samples examined	49	18											66 from 630 persons
Number of mixed samples + for *V. cholerae* (group I)	1												1
Number of mixed samples + for *V. cholerae* (group I) and vibrios (group II)													
Number of mixed samples + for non agglutinable vibrios (group II)	21	8											29
Number of mixed samples + for non agglutinable vibrios (groups II and III)													
Number of mixed samples + for non agglutinable vibrios (group III)	1												1
Number of single samples examined	200	631	726	898	1,077	1,646	1,478	1,312	2,105	1,234	2,269	1,794	15,370
Number of single samples + for *V. cholerae* (group I)		2			1				7	4	2	1	17 } 28
Number of single samples + for *V. cholerae* and vibrios (group II)	1	1		3	1		1	1	2	1			11 }
Number of single samples + for non agglutinable vibrios (group II)	23	52	68	35	20	14	23	53	31	29	33	42	423 446
Number of single samples + for non agglutinable vibrios (groups II and III)				8				1	1	1	1		12 } 533
Number of single samples + for non agglutinable vibrios (group III)	3	10	15	47	62	65	32	20	107	10	88	72	531 }

TABLE VIII

1933 *passengers*

	Jan	Feb	March	April	May	June	July	Aug	Sept	Oct	Nov	Dec	TOTALS
Number of persons supplying samples	379	400	379	385	561	537	452	368	419	174	429	293	4,776
Number of mixed samples examined	28	7											35 from 337 persons
Number of mixed samples + for *V cholerae* (group I)													
Number of mixed samples + for *V cholerae* and vibrios (group II)													
Number of mixed samples + for non agglutinable vibrios (group II)	3	1											4
Number of mixed samples + for non agglutinable vibrios (groups II and III)													
Number of mixed samples + for non agglutinable vibrios (group III)													
Number of single samples examined	112	330	379	385	561	537	452	368	419	174	429	293	4,439
Number of single samples + for *V cholerae* (group I)													
Number of single samples + for *V cholerae* and vibrios (group II)													
Number of single samples + for non agglutinable vibrios (group II)	3	4	6	4	4	3	1	1		1	3	2	31
Number of single samples + for non agglutinable vibrios (groups II and III)													
Number of single samples + for non agglutinable vibrios (group III)	1		2	1	6	3	1	2	4		3	1	24

TABLE IX

1934 *estate labourers*

	Jan	Feb.	March	April	May.	June	July	Totals.
Number of single samples examined	1,250	1,840	1,012	1,499	1,468	475	1,081	8,625
Number of single samples + for *V. choleræ* (group I)	1			1	2	2		6 } 13
Number of single samples + for *V. choleræ* and vibrios (group II)		3	1	3				7 }
Number of single samples + for non agglutinable vibrios (group II)	57	114	81	82	118	4	19	475 } 496
Number of single samples + for non agglutinable vibrios (groups II and III)	1	3	3	5	2	.		14 }
Number of single samples + for non-agglutinable vibrios (group III)	46	34	47	97	51	22	7	304 } 318

1934 *passengers*

	Jan	Feb.	March	April	May.	June	July	Totals.
Number of single samples examined	164	292	155	216	316	167	22	1,332
Number of single samples + for *V. choleræ* (group I)					1			1
Number of single samples + for *V. choleræ* and vibrios (group II)								
Number of single samples + for non agglutinable vibrios (group II)	2	3		3	3	1		12
Number of single samples + for non agglutinable vibrios (groups II and III)		.						
Number of single samples + for non agglutinable vibrios (group III)	1	2			2			5

TABLE X

	TOTALS AND PERCENTAGES FOR MIXED SAMPLES						TOTALS AND PERCENTAGES FOR SINGLE SAMPLES				PERCENTAGES FOR ALL SAMPLES	
	ESTATE LABOURERS			PASSENGERS			ESTATE LABOURERS		PASSENGERS		ESTATE LABOURERS	PASSENGERS
	Totals	Percentage per sample	Percentage per person	Totals	Percentage per sample	Percentage per person	Total	Per cent	Total	Per cent	Per person	Per person
Number of persons supplying samples	58,506			4,615								
Number of mixed samples examined	6,008			483								
Average number of single samples in the mixed samples		9 62			9 55	-						
Number of single samples examined							27,052		10,623			
Number of times *V cholerae* was isolated	36	0 59	0 062				45	0 16	3	0 023	0 094	0 019
Number of times non agglutinable vibrios of group II were isolated	1,555	25 8	2 6	30	6 2	0 04	1,176	4 3	94	0 88	3 19	0 81
Number of times non-agglutinable vibrios of group III were isolated	319	5 3	0 54	5	1 0	0 1	996	3 6	54	0 50	1 54	0 38

CHOLERA 'CARRIERS' ENTERING CEYLON YEARLY

The term 'carrier' is used in this paper to indicate any person who harbours agglutinable *V cholerae*, without prejudice as to whether or not that person can infect others with cholera

V cholerae was isolated on 36 occasions from 6,008 mixed samples from 58,506 estate labourers (*vide* Table X), that is one person in 1,627 was found to harbour this vibrio, but *V cholerae* was isolated on 45 occasions from 27,052 samples from estate labourers examined singly, that is one person in 601 by this method was found to harbour *V cholerae*   It is concluded that the difference between the figures 1,627 and 601 indicates the degree of error introduced by examining a number of samples mixed together

A somewhat similar degree of error is shown by the figures for the non-agglutinable vibrios   Twenty-seven thousand odd samples is a sufficient number for statistical purposes   Therefore, the conclusions in this paper are drawn more from the results of the examinations of single samples than of mixed samples

The number of other 3rd class passengers from whom single samples were examined was 10,623 and 3 were carriers (*vide* Table X), which was one in 3,541

The methods which were adopted for the isolation of vibrios have considerable limitations, because the vibrios may be present in insufficient numbers for successful isolation from culture media inoculated with only 0 1 gramme of fæces   It is well known that failure to isolate the vibrio from a person may be followed by successful isolation from the same person on subsequent days, and this was experienced in Ceylon during the outbreaks of 1925 and 1926   Therefore, whilst it has been shown definitely that one estate labourer in 601 and one passenger in 3,541 was a 'carrier', it is certain that the number of carriers was much greater than this

NON-AGGLUTINABLE VIBRIOS OF GROUP II

Vibrios of group II were isolated on 1,555 occasions from 6,008 mixed samples supplied by 58,506 estate labourers and this shows that at least 2 6 per cent of estate labourers harbour these vibrios   They were isolated on 1,176 occasions from 27,052 single samples, and this shows that at least 4 3 per cent of estate labourers harbour these vibrios   The difference in these percentages represents the degree of error between the methods of examining mixed samples and single samples, the degree of error is similar though smaller than for agglutinable *V cholerae*

The vibrios of group II were isolated on 94 occasions from 10,623 samples from other 3rd class passengers, which is 0 88 per cent

The number of estate labourers compared with the number of passengers who harboured these vibrios, was approximately in the proportion of 5 to 1 The proportion for the agglutinable *V cholerae* was 6 to 1, which is very similar

Seasonal prevalence —The vibrios of gioup II show an inteiesting seasonal prevalence

The numbei of occasions on which they were isolated was low during the months of June to Octobei—the diy season , the number inci eased duiing Novembei, and was high for the months of Decembei to April , the numbei usually fell during May to reach its lowest in June

Excluding the months of Novembei and May, Table XI compaies the pievalence of the two peiiods of five months, namely June to October and December to April The figures aie based on the iesults of the examinations of *single samples only* —

<div align="center">TABLE XI</div>

Summary of the seasonal pievalence of vibiios of gioup II foi the years 1931 to 1933

Season	Estate labourers			Passengers		
	Samples	Number positive	Percentage positive	Samples	Number positive	Percentage positive
June to October	4,352	393	9 0	3,682	56	1 5
December to April	8,735	164	1 8	4,143	14	0 33

THE CORRELATION BETWEEN THE PREVALENCE OF NON-AGGLUTINABLE VIBRIOS WITH THE PREVALENCE OF CHOLERA AND RAINFALL IN THE MADRAS PRESIDENCY

These correlations are shown in Graph 2 The upper curve shows the percentage of estate labourers and passengers who were found to harbour the vibrios of group II in all examinations, *that is foi both mixed and single samples* for each month from January 1931 to June 1934

The second curve shows the number of cases of cholera (in thousands) iecorded for the Madras Presidency during the same period

The third curve shows the rainfall in inches for the south-east of the Madras Presidency as recorded in the meteorological reports for India under ' Madras South-East '

The lowest curve shows the months when agglutinable *V cholerœ* were isolated , the heights of the thick black lines aie varied to show the relative number of times

the vibrios were isolated, but the monthly numbers are too small for the different heights of the black lines to be of much significance, they serve principally to show the dates when the agglutinable V *choleræ* were isolated

GRAPH 2.
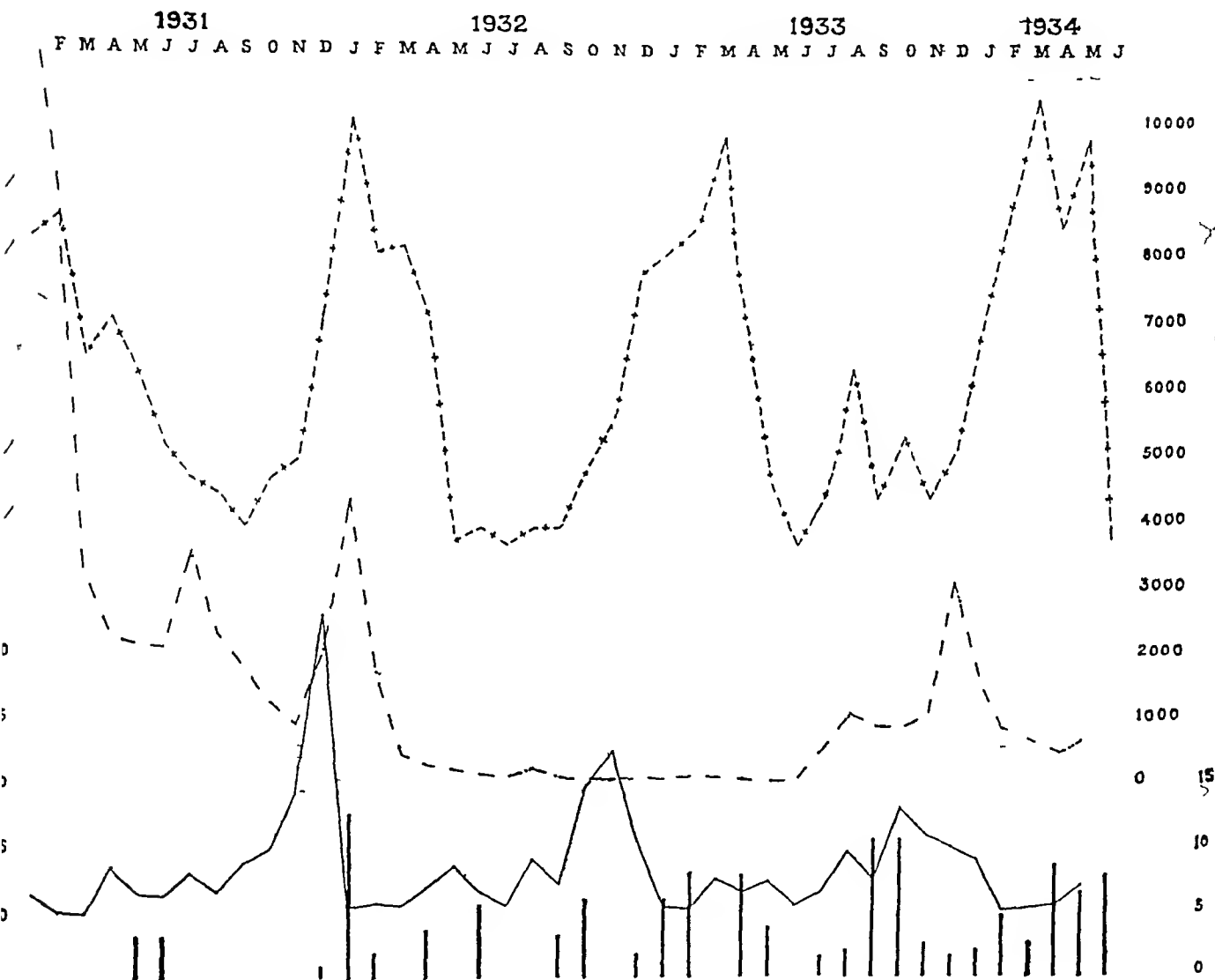
NON-AGGLUTINABLE VIBRIOS OF GROUP III

As already stated the vibrios of group III were not closely studied, it is probable that they comprise a number of species   Graph 3 shows the monthly percentage of estate labourers and passengers who harboured vibrios of group III for the period 1st January, 1931, to 30th June, 1934.

It will be seen that there is little or no correlation between the curves for these vibrios and the curves for vibrios of group II, the monthly incidence of cases of cholera or rainfall in the Madras Presidency as shown in Graph 2

GRAPH 3
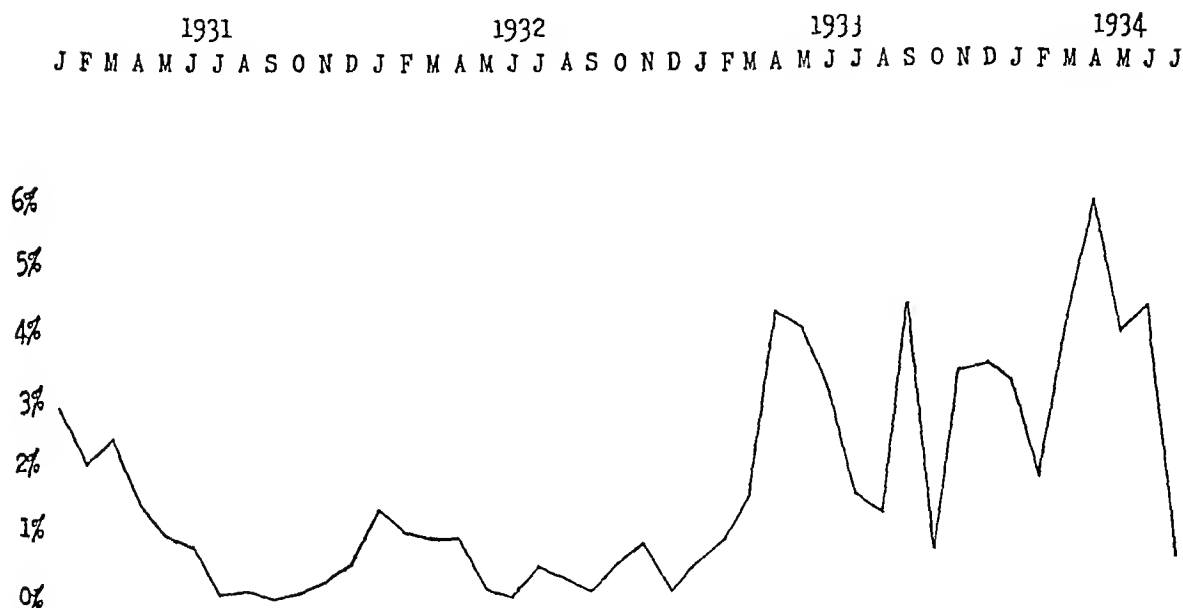
CHOLERA IN INDIA

There were approximately 217,000 deaths from cholera reported annually in India for the decade 1920 to 1929, and 31,000 of these were reported annually for the Madras Presidency   The population of the Madras Presidency was about 46 millions and that of Ceylon about $4\frac{1}{2}$ millions for that decade, and if the prevalence of the disease was the same for the two countries the expected annual death rate would be over 3,000 for Ceylon

CHOLERA IN CEYLON

The population of Ceylon is well provided by the State with medical officers for treatment and sanitation

There are general and infectious diseases hospitals in or near Colombo with 1,266 beds , and in other parts of Ceylon there are 89 general hospitals with 6,506 beds   There are 626 government dispensaries in the island, and apart from these there are municipal dispensaries and 86 estate dispensaries

The sanitation of the island is looked after by 43 medical men, including 25 medical officers of health, and working under these are 267 well-trained sanitary inspectors   (This does not include the municipalities)

It may be concluded from this that cases of cholera are unlikely to go unrecognized

Many samples of stools from cases of enteritis are sent for examination to the Bacteriological Institute in Colombo

There are twenty large files of minutes and reports in the government medical offices in Colombo which are records of what has been done in connection with outbreaks of cholera for the years 1925 to 1933, these have been closely studied for this paper

When a case of cholera has been reported the medical officers of health, the provincial surgeon the district medical officer, the headman of the district sanitary inspectors and police have been mobilized. Within 24 hours the patients and all contacts have been isolated, traffic from the area has been stopped, water-supplies have been guarded and the necessary disinfection carried out. The antecedents of all cases have been recorded

Dr S F Chellappah, a senior officer of the sanitation department, has taken charge of the larger outbreaks, and has written long reports well illustrated with photos, maps, graphs and tables. He has traced the source of infection in the explosive outbreaks to a definite well or other water-supply, and in other cases has traced the infection from one patient to another. He has failed in very few cases to discover the source of infection of each patient. Rectal swabs for bacteriological examination were taken from most of the contacts in these outbreaks

Ceylon may be divided for the purposes of this study of past records into three areas —

Area I in which the average yearly rainfall is from 100 to over 200 inches (*vide* Map 2). This area is montane and submontane, and the recruited estate labourers are sent to this area where all the tea and rubber estates are located

Area II in which the average yearly rainfall is from 75 to 100 inches. This is the low country wet zone

Area III in which the average yearly rainfall is 75 inches or less. This is the low country dry zone

Cholera has occurred on 30 occasions in the nine years under consideration. On 21 occasions only one person was affected, and the other 9 were outbreaks in which three or more persons acquired cholera

The following is a précis of the records concerning these 30 occasions —

AREA I Cholera has occurred in this area on ten occasions, on seven of these only one person was affected, all of these had just arrived in Ceylon after being quarantined at Mandapam camp, five had entered the camp seven days, one eight days, and one nine days prior to the onset of the disease

The other three were small outbreaks involving 4, 7 and 13 persons respectively

Two of these were started by recent arrivals from South India, they were estate labourers who had entered Mandapam camp 7 and 8 days respectively prior to the first symptoms. The source of the largest outbreak was never discovered. The bacteriological findings were positive on all these occasions

AREA II Cholera occurred in this area on nine occasions, and in each case only one person was affected. Three were 3rd class passengers who became ill a day

CEYLON

SHOWING

ANNUAL AVERAGE RAINFALL

| | 25 50 INCHES |
| 50 75 |
| 75 100 " |
| 100-125 " |
| 125 150 " |
| 150 200 " |
| > 200 |
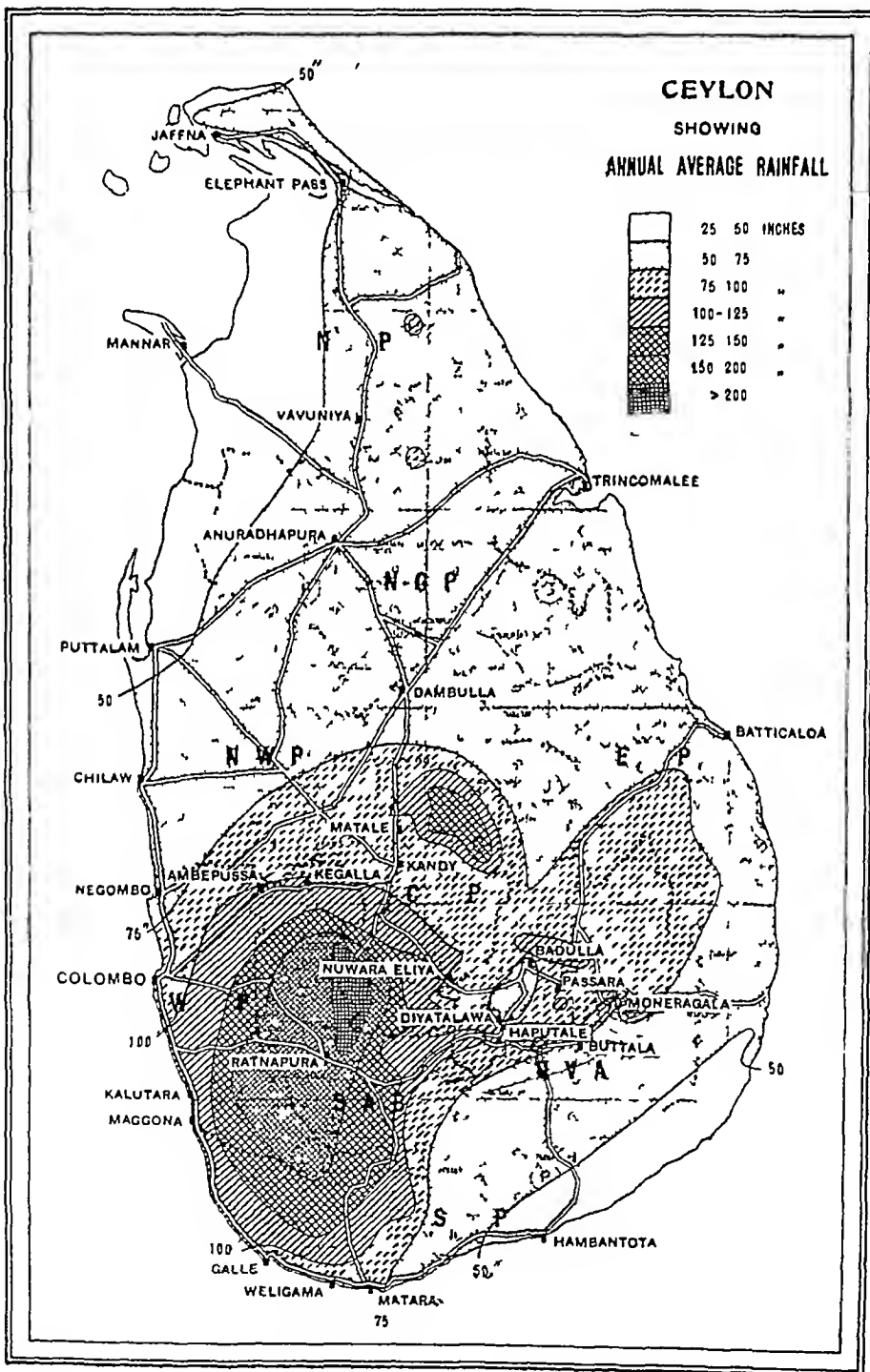
or two after arrival from Southern India   The patients on the other six occasions were permanent residents and the source of infection was never traced

AREA III   Cholera occurred in this area on 11 occasions, five of these were in persons who had recently landed from Southern India, and six were outbreaks, which were of considerable size for Ceylon, because the total reached 349 cases

Five of these outbreaks took place in 1925 and 1926   In two of them the first patients were seamen who developed the disease while returning to the northern province of Ceylon in sailing boats which had been to the pearl fisheries off Southern India   The other three outbreaks of 1925 and 1926 started during a time when the Admiralty were undertaking large building operations at Trincomalie and the Government of Ceylon was building two railways, one connecting Trincomalie, and the other connecting Batticaloa to the main line   Consequently there was a considerable influx of labourers from South India, and although the original infectors of these outbreaks were never traced undoubtedly they came from Southern India

The sixth outbreak started in December 1928 in the small town of Alutnuwara, where there is a shrine, whither many pilgrims travel, but it is curious that the outbreak did not occur in the season of pilgrimage

The following are the number of notified cases of cholera for the twelve years 1922 to 1933 —

1922	1923	1924	1925	1926	1927	1928	1929	1930	1931	1932	1933
Nil	*Nil*	4	305	56	6	4	19	*Nil*	4	*Nil*	*Nil*

CHOLERA IN MANDAPAM CAMP

On 14 occasions persons in the incubation stage of cholera developed the disease in Mandapam camp

On three occasions another person was infected presumably by contact, while on one occasion, in October 1925, an outbreak resulted in the infection of ten persons

THE CIRCUMSTANCES OF THE ESTATE LABOURERS BEFORE AND AFTER THEY LEAVE INDIA

The lowlands of south-east India are watered by the rains of the north-east monsoon , the rains of the south-west monsoon pass by them

Towards the end of the dry season the lands have become arid, green vegetation disappears, the river beds have dried up or maintain only a trickle of water, the tanks are empty or hold a little muddy water, and the inhabitants depend upon the shrunken water of their wells   The sites of these wells have not been selected, nor the construction of them done with a view to the avoidance of contamination

The diets of the poorest classes are never of a high quality, and become deficient, especially in vitamins, towards the end of the dry weather when fresh foods are scanty or unprocurable   When the rains of the north-east monsoon are below normal the indigent among the population starve

The villagers live in huts which are dark, insanitary and hardly weatherproof The north-east monsoon rains find them with lowered resistance following a time of want, and the conditions in the villages are such that pollution and re-pollution of their water-supplies take place with each shower of rain

As soon as the labourers, living under these conditions, are recruited for work in Ceylon their interests are watched by the Governments of Ceylon and India

There are ordinances which provide for a minimum wage, the amount and quality of rice which must be issued to each labourer, the housing accommodation, medical inspection, medical treatment and the necessary powers for the inspection of their circumstances and inquiries into any grievances   Schools are provided for the children, and crèches for the care of the children during the working hours of their parents are to be found on some estates

Latrines are provided and the water-supplies on the estates are protected wells or pipes from dammed-up streams in the higher hills   The liability of pollution is remote in most cases

Mr M W M Yeattes of the Indian Civil Service subscribes to the improvement of the circumstances of these labourers after they travel to Ceylon from the plains of Southern India (vide *Census of India*, Volume XIV, 1931, Madras, Part I, pages 93 and 94)   He states —

' Emigration is a great teacher of self respect, for caste is to a large extent put away when the Indian emigrant crosses the sea   One social effect of emigration has been indicated above, viz , a growth in independence and self respect on the part of the depressed classes who go abroad   This is all to the good A man who, little removed from predial serfdom in Tanjore, finds himself treated on his own merits like every one else when he crosses the sea, paid in cash for his labours, and left to his own resources, must in the majority of cases benefit from the change, and it is probably the existence of the emigration current that has contributed most to the growth of consciousness among the depressed classes in India   Labourers from well-run estates generally bring back to their villages some of the ideas on cleanliness, food and comfort acquired while abroad Evidences of this are seen in many a South Indian village, and I have myself on several occasions had pointed out to me a house differing markedly from its neighbours as being that of some one who had been to Malaya or Ceylon '

The Government of India have stationed in Ceylon an agent to guard the interests of their aforetime subjects in their improved circumstances

The death rate among the estate labourers is under 20 per 1,000, which is lower than that of the general population of Ceylon, and still lower than that of the inhabitants of the areas in India from which they have come

Now this comparative state of affairs in India and Ceylon must be taken into account when considering the incidence of such a disease as cholera

LENGTH OF TIME THE AGGLUTINABLE *V choleræ* SURVIVES IN ' CARRIERS '

The problem of the duration of time a healthy person infected with agglutinable *V choleræ* continues to harbour the organism has been investigated by many workers, and the results of their work show that the duration is usually short

The majority of carriers cease to excrete *V cholerae* after six to eight days, a fair number continue this excretion for a longer time, up to about three weeks, and a few continue to discharge them for two or three months

A few carriers have been detained at Mandapam camp and their stools examined daily for *V cholerae* But the results are of little value because it was unknown when these persons became infected

In thirteen cases in which only agglutinable vibrios had been isolated on the first examination six were still excreting them on the fifth day, and seven were negative, but unfortunately the intermittent manner in which these vibrios are excreted was not taken into account and in most cases when a sample was negative no further examinations were done It is of interest that the laboratory notes from Mandapam camp show that non-agglutinable vibrios appeared in five of the thirteen cases at or about the time when the agglutinable vibrios disappeared

During the outbreak in the north of Ceylon in 1926, rectal swabs were taken daily or bi-daily from thirteen carriers, who were isolated in the contact camps The day on which these persons became infected could be presumed with considerable accuracy, and the examination of swabs from them was continued for several days after they had first become negative The following table gives the results —

<div align="center">

TABLE XII

Day after infection	Negative	Positive
3rd	3	10
5th	4	9
6th	7	6
9th	10	3
11th	11	2

</div>

The subsequent history of the two who were positive on the 11th day is One was negative on the 12th day and positive on the 13th and thereafter negative The other was negative on the 13th and 16th days and positive on the 19th and 21st days and thereafter negative

<div align="center">

DISCUSSION

The virulence of the agglutinating V *cholerae of ' carriers '*

</div>

The labourers and passengers (examined for carriers) were fair samples of the emigrants who pass yearly from South India to Ceylon

When the figures already given are used as a basis from which to calculate the number of persons who were carriers three days before they reached Ceylon Table XIII gives the result —

<div align="center">

TABLE XIII

Calculated number of 'carriers'

</div>

Travellers to Ceylon	1931	1932	1933	Average for years 1924 to 1933
Estate labourers	68,337	50,869	32,898	102,305
Other 3rd class passengers	100,854	92,895	88,353	109,825
CALCULATED NUMBER OF CARRIERS—				
Among estate labourers	113	83	54	170
Among 3rd class passengers	28	26	25	31
TOTALS	141	109	79	201

The years 1931 to 1933 were years of depression, and the result was that fewer labourers travelled to Ceylon Therefore (as Table XIII shows) the calculated number of 'carriers' was low for these years

It has been shown that cholera occurred on ten occasions during the years 1925 to 1933 in the areas to which the estate labourers go and that on nine of these occasions the disease was due to a recent arrival being in the incubation period

The number of carriers will have greatly diminished a few days after their advent to Ceylon, and by the end of three weeks few, if any, will continue to be carriers This and the improved environment and circumstances of the labourers might be held to account for the rarity of cholera among them But their habits are such that contact infection would occur if virulent vibrios were being disseminated by a number of them Therefore it is concluded that the majority of the agglutinating vibrios of carriers are of very low virulence

This is strongly supported by the nature of the journey they make to Ceylon which places them under circumstances for infection by contact to be easily acquired

Many of these labourers travel in large batches under the charge of those who have recruited them

They are packed into third class carriages at Mandapam and two hours later are transferred to the decks of the ferry boats at Dhanushkodi The sea of the straits between India and Ceylon can be rough, and these labourers are very susceptible to sea-sickness and a common sight is a number of them with their wives and children collapsed cheek by jowl upon the decks and rolling on to one another with every movement of the boat The decks soon become polluted with vomit and other excrement, and where more educated people will struggle with the sickness sufficiently to reach the lavatories or the side of the boat, many of these labourers and their dependents abandon themselves to a state of collapse and make no attempt to avoid polluting the decks

When they arrive at Talaimannar about two and a half hours later they are crowded into third class carriages for a night journey to the interior of Ceylon The journey by the time they reach the estates has occupied at a minimum 20 hours

Since no case of cholera has occurred in Ceylon for the last ten years in which it could be assumed that the patient had acquired the infection on the journey, it is strong evidence for the low virulence of the agglutinating vibrios of carriers

The result of this investigation might be held to support the views of those who believe that healthy persons harbouring agglutinable *V choleræ* are never a source of danger to others d'Herelle, Malone and Lahiri (1930) are emphatic on this matter and state ' Only those suffering from cholera and in the incubation stage are infective '

Such a final determination of the potentialities of an agglutinating *V choleræ* for its normal host is not justifiable in the present state of our knowledge

There is very little evidence of carrier infection to be found in the literature In a country where cholera is endemic it would be very difficult or impossible to incriminate a carrier, because choleraic diarrhœa is not uncommon , and carriage on food, clothes or by flies cannot be excluded easily

A letter has been received, dated 18th March, 1932, from the Director, League of Nations, Health Organization, Eastern Bureau, Singapore, in which the following statement occurs :—

' Professor Ciuca last week related two instances which had come under his own notice where carrier infection seemed definite One was the case of a boy who had made a journey from his home in Roumania with his father On the return journey the father died of cholera All the necessary precautions against spread were taken by the sanitary authority of the village in which the death occurred The boy proceeded to his home, but within a very short period of his arrival there both his mother and sister developed cholera As there was no cholera at all in the village there seemed no other solution of the question than that the boy was himself a carrier and this proved to be the case

The second instance occurred during the second Balkan War, when a serious cholera epidemic occurred among Roumanian troops At the conclusion of hostilities all Roumanian troops were ordered to proceed to Lazarets on the frontier before re-entering their own country This was carried out in all cases except one, where a small detachment crossed into Roumania without examination

Following the return of this detachment an outbreak of cholera developed, due definitely to carrier infection'

Therefore, although it may be concluded that the vibrio in the majority of carriers is of low virulence, it cannot be concluded that there is never an exceptional carrier who harbours a vibrio of high virulence, such a person would probably have a high resistance to the disease

Another aspect of this question must be considered   It is accepted that the virulence of certain organisms can be enhanced by serial passage through selected animals and this may be made to occur quickly when the resistances of the animals have been lowered

Even if a fairly well-fed population living under sanitary conditions is at little risk from the presence of carriers, such as those coming to Ceylon, because the vibrios harboured by them are not infective on their first passage, yet it is probable, given the circumstances of frequent passage from one ill-nourished person to another, that a sufficient enhancement of virulence will take place to start an epidemic   And it may be that the epidemics in Ceylon in 1925, the sources of which defeated such an expert tracker of the first case as Dr Chellappah, originated in this way

The nature of the vibrios of group II

Zlatogoraff (1911) appears to have been the first to put forward the view that the vibrios frequently found in stools during cholera epidemics and which differed from *V choleræ* only in being non-agglutinable were true cholera vibrios which had lost agglutinability   Horowitz (1911) supported this view, others opposed it   Crendiropoulo (1913) working in Egypt among pilgrims at the camp of the Sanitary Maritime and Quarantine Council also considered that these inagglutinable vibrios were true cholera vibrios   More recently the same opinion has been expressed by Tomb and Maitra (1926), Pasricha, de Monte and Gupta (1931) and by d'Herelle *et al* (*loc cit*)

The results of the work at Mandapam camp and an investigation during the outbreak of cholera in 1926 which support this view are —

(a) Ten of the cultures which on first isolation were agglutinable in high titre have become non-agglutinable and are indistinguishable from the vibrios of group II

(b) On 21 out of 84 occasions on which agglutinable vibrios were isolated, non-agglutinable vibrios also were isolated   Such a high percentage of non-agglutinable vibrios was never shown for any other group of labourers during these researches

(c) On 13 occasions agglutinable vibrios only were isolated from the first samples of stools of the carriers   When these agglutinable vibrios could no longer be isolated on five of these 13 occasions they were replaced by non-agglutinable vibrios

(d) The outbreak of cholera in 1926 in the north of the island was confined to the Jaffna peninsula   There were in all 48 definite cases of cholera and 46 of the patients died   Thirty-four of these cases occurred in Jaffna town of which 17 were among prisoners in the jail

A motor laboratory van is kept in readiness at the Bacteriological Institute in Colombo, so that it may be dispatched immediately for the investigation of outbreaks of cholera, plague, etc, in distant parts of Ceylon It was built for the safe carriage of all necessary laboratory equipment and stores The van was sent to Jaffna in the charge of Mr K Vallipuram, the senior assistant of this laboratory, and his work consisted in examining any material sent to him by the medical officers working in connection with the outbreak Rectal swabs were taken from all the prisoners, from the contacts in the isolation camp, and also from sections of the population, including hospital patients, who were considered not to have been in contact with the infection Table XIV shows the results of the laboratory examinations —

TABLE XIV

	Number of swabs	Number positive for non-agglutinating vibrios	Percentage positive
Prisoners ..	286	40	14
Camp	230	24	10 4
Persons not con tacts as far as was known	214	4	1 5

(e) From 21st April to 26th April when cases of cholera were occurring in the Jaffna jail, there were 34 cases of diarrhœa with bile-stained stools and no symptoms of cholera These were never recorded as cases of cholera, although rectal swabs from these patients were sent for bacteriological examination with the following results —

Agglutinating vibrios were isolated from   8 swabs
Agglutinating vibrios and non-agglutinating vibrios from 2 swabs
Non-agglutinating vibrios from   ..   . 14 swabs

Thus 24 patients harboured either agglutinating or non-agglutinating vibrios

(f) The examination of rectal swabs of the contact prisoners in Jaffna jail started on 30th April and the laboratory records show that at first many agglutinating vibrios were isolated, and not many non-agglutinating vibrios, but later when the agglutinating vibrios were appearing many non-agglutinating appeared, and these in their turn were disappearing at the end of May and beginning of June when the work ceased

The prevalence of vibrios of group II is shown in Graph 2 to increase during the cholera season in the Madras Presidency, but this cannot be brought forward as strong evidence that the vibrios are *V cholerae* which have lost their agglutinability, because it may be that the conditions which spread *V cholerae* will also spread other vibrios

It has already been shown that the other 3rd class passengers, who are quarantined at Mandapam, harbour vibrios much less frequently than the estate labourers

These passengers are from many classes, some are Mohammedan and Hindu traders, others are going for domestic service in Ceylon, and a number of them are non-recruited labourers seeking work on the estates and in the towns of Ceylon

Unfortunately no records were kept of the status of those who were found to harbour vibrios , and it is very probable that the vibrios were isolated from the lower caste labourers and not from the better class persons among these passengers, who do not come from villages where cholera is endemic

But taking all the evidence into consideration it is difficult to avoid the conclusion that the vibrios of group II are non-agglutinable avirulent *V cholerae* Or to put it in another way, it may be that the vibrio of cholera is the virulent variant of a vibrio commonly occurring in the intestines of people living under extremely insanitary conditions

SUMMARY

(1) An investigation of 100,896 estate labourers and passengers, who were quarantined before entering Ceylon, resulted in the discovery of 84 carriers of agglutinating *V cholerae*

(2) A calculation from the figures indicates that at least 200 persons in a year of average emigration are carriers three days before they arrive in Ceylon Many will continue to be carriers for variable periods after entering Ceylon

(3) It is shown that there were 10 occurrences of cholera during the last nine years in the areas to which the majority of the carriers go, and that nine out of these 10 occurrences were due to persons in the incubation period On the other occasion the source of the infection was not traced This and other evidence put forward indicates that the great majority of carriers have been excreting avirulent vibrios

(4) The number of persons excreting non-agglutinable vibrios was investigated, these vibrios are placed into one of two groups—those which resemble *V cholerae* in all respects except agglutinability, and those which differ markedly from *V cholerae*

(5) It is shown that, during the cholera season in the Madras Presidency, the vibrios resembling *V cholerae* may occur in 10 per cent of the estate labourers But during the dry season the prevalence of them falls to 1 or 2 per cent

(6) The relationship between the non-agglutinable vibrios and the true cholera vibrio is discussed

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BIOCHEMICAL INVESTIGATIONS ON DIFFERENT VARIETIES OF BENGAL RICE.

Part I

THE CHEMICAL COMPOSITION OF VARIOUS RICE SAMPLES

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THE chemical composition of rices grown in some parts of India has been determined by previous investigators   Sen (1916) has analysed some varieties of Bihar rice, while McCarrison and Norris (1924) have done similar work on South Indian rice   Recently Joachim and Kandiah (1928) have undertaken an analysis of Ceylon rice   Bengal is one of the largest rice-producing provinces where as many as 540 different varieties of rice are known (Hector *et al* , 1934)   There is, however, no systematic analysis of Bengal rice on record

Bengal rice may be divided into three main classes, viz , Aus, Aman and Boro It is a common belief, and this belief is shared even by some leading medical authorities (Acton and Chopra, 1925), that Aus rice is inferior to Aman practically in all respects   While chemical analysis might throw some light on this point, a thorough and complete biochemical investigation is necessary before it is possible to select the better varieties

The present paper forms part of a systematic biochemical investigation of Bengal rice taken up by the present authors   The investigation will include chemical analysis, determination of enzymic digestibility of proteins and carbohydrates, isolation, analysis and determination of biological value of proteins determination of vitamin content and examination of sterols of the different varieties

All the varieties of Aus and Aman rices used in this work were very kindly supplied by the Economic Botanist to the Government of Bengal from his test plots While these were mostly pure-line strains, there were two hybrids as well A short description of the paddies might not be out of place

DESCRIPTION OF THE VARIETIES USED

Aus—

1   *Charnock* (Dacca No 6) —A very fine, high land Aus paddy ripening earlier than Dacca Nos 2 and 4   For a combination of yield and fineness it is one of the best paddies

2   *Dhairal* (Dacca No 14) —A coarse high land Aus paddy of almost the same duration as Dacca No 2 and suitable for the same type of land as Dacca Nos 2 and 4   It is a very heavy yielder and is liked by the cultivators for their own use

3   *Surjamukhi* (Dacca No 4) —A medium fine, high land Aus paddy very similar in all respects to Kataktara

4   *Kumari* (Dacca No 16) —A medium fine, high land paddy of good quality and suitable for the same type of land as Dacca Nos 2 and 4

5   *PXS* (8) (Dacca No 18) —A strain of Aus hybrid   It is very early and is particularly suited for rich light soils and also for double-cropped lands

6   *Bhutmuri* (Bankura No 4) —An improved heavy yielding strain of Aus paddy of Bankura known as Bhutmari   It is suitable for higher situations and is drought-resistant   It yields a coarse rice which is liked by the cultivators and labouring classes

7   *Jhanji* (Bankura No 2) —A pure-line strain of transplanted Aus paddy of Bankura   It is coarse, heavy yielding and suitable for higher situations of transplanted paddy lands

8   *Kataktara* (Dacca No 2) —A medium fine, broadcast, high yielding Aus paddy taking about four months to mature   It does best on fertile high lands in rotation with a 'rabi' crop

Aman—

9   *Indrasail* (Dacca No 1) —A medium coarse, transplanted Aman paddy, ripening normally about 14th December   It is very suitable for areas in North and East Bengal, where the soil is fertile and moisture lasts up to November   It should not be grown on ordinary double-cropped lands

10   *DXI* (34) (Dacca No 21) —An early strain of Aman hybrid   It is a paddy of very superior quality and a very heavy yielder

11   *Dudshar* (Dacca No 5) —This paddy is similar in all respects to Dacca No 1 but ripens a bit earlier

12   *Badkalamkati* (Bankura No 1) —An improved strain of Badkalamkati paddy of Bankura belonging to the 'kartik' paddy group   It is a paddy of good quality, ripening very early and is fairly heavy yielding

13   *Lalisail* (Dacca No 17) —An oval-shaped coarse, heavy yielding paddy as early as Dudshar and grows in same types of lands as are suited to Indrasail and Dudshar   It can also be grown on double-cropped lands

14   *Bhasamanik* (Chinsurah No 2) —A fine, selected pure-line variety of West Bengal   It is a heavy yielder and has beaten all other varieties in the Chinsurah farm   It is an ideal paddy for general use combining yield with quality

15   *Jhingasail* (Dacca No 15) —A finer paddy than Indrasail or Dudshar It ripens about the same time as Dudshar and yields the same amount under identical conditions

16   *Nagra 68/6* (Chinsurah No 1) —A selected heavy yielding strain of the Nagra paddy of West Bengal   It is of medium quality and suitable for the same type of lands as Dacca No 1

17   *Khiraiyah* (Barisal No 2) —An improved strain of Balam paddy of Barisal district   It is of good quality and suitable for the Balam tract of Bakerganj district

18   *Chingrighusi* (Barisal No 1) —An improved heavy yielding strain of Balam paddy of Barisal having similar qualities as Khiraiah

PREPARATION OF SAMPLES

Samples having different treatments were all prepared in the laboratory under controlled conditions

1   *Husking* —A quantity of paddy was placed in an indigenous wooden mortar and pestle known in East Bengal as ' kahal chia ' and by slight rotatory movement of the pestle the paddy was unhusked with practically no loss of the coloured skin

2   *Polishing* —Where polishing was necessary the husked rice obtained above was further treated in the mortar with up and down movement of the pestle until the desired polished stage was obtained

3   *Parboiling* —Parboiled samples were prepared as usual by allowing a quantity of paddy to be steeped in water over-night and then boiling with minimum quantity of water till the desired stage was obtained, followed by drying in the sun

4   *Bran* —The rejected portion in the process (2) is the bran

5   *Husk* —The rejected portion in the process (1) is the husk

EXPERIMENTAL

A large number of paddies and rices was analysed as detailed below —

Paddies —All the Aus and Aman varieties described before

Rices —(a) Sun-dried and non-polished } from all the Aus and Aman
            (b) Sun-dried and polished   } varieties described
            (c) Parboiled and non-polished }

Bran from—(a) Sun-dried Charnock and Jhanji
                 (b) Parboiled Charnock and Jhanji

Husk from—(a) Sun-dried Charnock and Jhanji
                 (b) Parboiled Charnock and Jhanji

METHODS OF ANALYSIS

Moisture, oil and fat, crude fibre, total nitrogen, protein (total nitrogen × 6 25), soluble carbohydrate, ash, silica and calcium were determined by methods recommended by the Association of Official Agricultural Chemists

Ferric oxide was estimated by the Zimmermann-Rheinhardt method (Treadwell, 1930)

Phosphoric acid and potash were estimated by the method of Neubauer-Finkener as described by Stewart

EXPERIMENTAL DATA

The experimental data are summarized in the following tables* Table I indicates the variations in chemical composition of paddies of Bengal, Table II that of sun-dried non-polished (husked) rices, Table III that of sun-dried polished rices, while Table IV indicates the variations in chemical composition of parboiled non-polished rices

A comparison of Tables I, II and IV will show that the effect of husking is to reduce greatly the content of woody fibre, ash and silica and to increase the content of carbohydrates, proteins and fats and to a smaller extent of phosphoric acid Husking has got no effect on the potash content which appears to be uniformly distributed throughout the grain

It will be seen from these tables that in their chemical composition, the individual Aus varieties show some deviation from the average value The same remark also holds good for different Aman varieties

Rice contains an excessive proportion of starch and therefore the relative nutritive value of a sample of rice depends on its content of proteins and mineral constituents, especially lime and phosphoric acid

Amongst the rices of Aus class Surjamukhi, Kumari and Kataktara are poorer with regard to their protein content, while Bhutmuri, Jhanji and Charnock varieties contain the maximum amount of protein Surjamukhi, Kataktara and Charnock show relatively higher fat content, while the ash as also the phosphoric acid and calcium contents of Charnock is the highest Polished sun-dried Surjamukhi is deficient in phosphoric acid

Of the Aman rices Latisail, Khiraijali and Dudshar appear to contain the maximum amount of protein, while Jhingasail contains the minimum amount Indrasail is relatively poor in fat

In the case of the two hybrids, the paddies of both PXS (8) and DXI (34) contain comparatively lower amounts of protein But the protein content of the rices derived from these paddies almost reaches the respective average values for Aus and Aman

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* The figures in all the succeeding tables are calculated on air dry basis The slight variations in moisture content do not affect the general conclusions

TABLE I

Showing the variation in chemical composition of paddies of Bengal (expressed as per cent on air-dry basis).

	Variety	Moisture	Fat	Protein	Woody fibre	Soluble carbohydrate	Ash	Total nitrogen	Silica	P_2O_5	K_2O	CaO	Fe_2O_3
	Aus												
1	Charnock	9 9	1 30	6 94	10 12	63 70	8 04	1 11	6 71	0 74	0 30	0 053	0 017
2	Dhairal	10 2	1 11	6 63	9 75	66 41	5 90	1 06	4 40	0 56	0 29	0 051	0 016
3	Surjamukhi	10 1	1 32	5 89	10 05	67 15	5 48	0 91	4 80	0 65	0 35	0 028	0 016
4	Kumari	10 9	0 98	5 06	9 78	67 09	6 10	0 91					
5	PXS (8)	9 7	1 22	5 88	9 87	67 40	5 93	0 94	4 90	0 61	0 29	0 031	0 017
6	Bhutmuri	9 4	1 03	7 13	9 72	66 92	5 80	1 14	5 3	0 63	0 34	0 031	0 018
7	Jhaap	9 4	1 02	7 06	9 82	66 81	5 89	1 13					
8	Kataktara	10 5	1 24	5 25	9 81	66 90	6 30	0 84					
	Averages	*10 1*	*1 15*	*6 23*	*9 87*	*66 47*	*6 18*	*0 99*	*5 2*	*0 64*	*0 31*	*0 039*	*0 076*
	Aman												
9	Indrasail	10 5	1 15	7 00	9 70	66 83	4 82	1 12	3 8	0 49	0 37	0 068	0 016
10	DXI (34)	10 3	1 72	5 00	10 59	68 09	4 30	0 96					
11	Dudshar	10 4	1 30	6 38	10 15	67 48	4 20	1 02	3 2	0 48	0 38	0 070	0 016
12	Badkalamkati	9 8	1 68	6 19	10 51	67 72	4 10	0 99					
13	Latisail	10 5	1 91	7 44	10 17	65 71	4 59	1 19	3 6	0 51	0 39	0 065	0 015
14	Bhasamanik	10 5	1 79	6 19	10 55	66 57	4 40	0 99					
15	Jhingasail	9 8	1 67	5 63	11 00	66 83	5 07	0 90	4 1	0 58	0 36	0 031	0 014
16	Nagra 68/8	10 3	1 77	6 13	10 57	66 63	4 60	0 98					
17	Khirajali	10 6	1 84	7 06	10 19	65 87	4 44	1 13	3 4	0 56	0 39	0 061	0 016
18	Chungrighusi	10 7	1 80	6 25	10 61	66 14	4 50	1 00					
	Averages	*10 34*	*1 64*	*6 33*	*10 40*	*66 79*	*4 50*	*1 028*	*3 62*	*0 53*	*0 38*	*0 059*	*0 015*

TABLE II

Showing the variation in chemical composition of sun-dried husked rices of Bengal (expressed as per cent on air-dry basis)

Variety.	Moisture	Fat	Protein	Woody fibre	Soluble carbo-hydrate	Ash	Total nitrogen	Silica	P$_2$O$_5$	K$_2$O	CaO	Fe$_2$O$_3$
Aus												
1 Charnock	10 7	1 64	8 13	0 61	77 37	1 55	1 30	0 26	0 81	0 34	0 025	0 016
2 Dhairal	10 4	1 58	7 50	0 52	78 62	1 38	1 20	0 17	0 68	0 33	0 025	0 015
3 Surjamukhi	9 9	1 71	6 08	0 60	80 41	1 22	0 97					
4 Kumari	10 7	1 45	5 56	0 54	80 36	1 39	0 89	0 16	0 79	0 35	0 016	0 015
5 PNS (8)	10 2	1 61	7 50	0 58	80 41	1 36	1 20					
6 Bhutmuri	10 4	1 51	8 44	0 51	77 83	1 31	1 35					
7 Jhatji	10 2	1 51	8 44	0 57	78 02	1 26	1 35	0 15	0 81	0 31	0 019	0 016
8 Kataktara	10 2	1 68	5 75	0 56	80 33	1 48	0 92	0 25	0 72	0 35	0 019	0 016
Averages	*10 3*	*1 58*	*7 18*	*0 59*	*79 17*	*1 37*	*1 14*	*0 20*	*0 76*	*0 33*	*0 021*	*0 016*
Aman												
9 Indrasail	9 7	1 33	7 81	0 51	79 47	1 18	1 25	0 07	0 56	0 41	0 068	0 015
10 DXI (34)	10 1	2 04	7 63	0 71	78 51	1 01	1 22					
11 Dudhsar	9 9	2 25	8 81	0 63	77 34	1 27	1 39	0 06	0 61	0 45	0 071	0 014
12 Badkalamkati	9 6	2 45	7 25	0 65	79 01	1 04	1 16					
13 Latisail	9 5	2 33	8 25	0 64	78 04	1 24	1 32	0 07	0 58	0 46	0 065	0 014
14 Bhasmanik	10 3	2 31	7 63	0 67	77 97	1 12	1 22					
15 Jhingasail	10 0	2 31	6 81	0 81	78 86	1 21	1 09	0 08	0 63	0 41	0 032	0 015
16 Nagra 68/6	10 8	2 11	7 25	0 69	77 99	1 16	1 16					
17 Khirajpah	9 7	2 41	8 25	0 64	77 81	1 19	1 32	0 07	0 64	0 43	0 065	0 014
18 Chingrighar	9 9	2 35	7 63	0 75	78 25	1 12	1 22					
Averages	*9 95*	*2 19*	*7 71*	*0 67*	*78 32*	*1 15*	*1 24*	*0 07*	*0 60*	*0 43*	*0 06*	*0 014*

TABLE III

Showing the variation in chemical composition of sun-dried polished rices of Bengal (expressed as per cent on air-dry basis)

	Variety	Moisture	Fat	Protein	Woody fibre	Soluble carbo hydrate	Ash	Total nitrogen	Silica	P_2O_5	K_2O	CaO	Fe_2O_3
	Aus,												
1	Charnock	10 9	1 22	7 50	0 26	78 85	1 21	1 21	0 26	0 63	0 25	0 020	0·014
2	Dhenral	11 4	1 01	7 13	0 17	79 38	0 91	1 14	0 18	0 36	0 16	0 020	0 013
3	Surjamukhi	10 7	1 28	5 81	0 24	81 71	0 76	0 93					
4	Kumari	11 8	0 81	5 19	0 21	81 17	0 82	0 83	0 15	0 44	0 20	0 014	0 013
5	PXS (8)	11 7	1 11	7 19	0 23	78 19	0 86	1 15					
6	Bhutmuri	11 4	0 91	8 00	0 18	78 81	0 70	1 28	0 14	0 42	0 14	0 017	0 014
7	Jhanji	11 1	0 91	7 94	0 22	79 22	0 61	1 27	0 15	0 40	0 21	0 014	0 013
8	Katuktara	10 8	1 12	5 31	0 20	81 75	0 82	0 85					
	Averages	*11 2*	*1 04*	*6 76*	*0 22*	*79 98*	*0 83*	*1 08*	*0 18*	*0 45*	*0 19*	*0 017*	*0 013*
	Aman												
9	Indrasail	11 4	1 03	7 19	0 15	79 62	0 61	1 15	0 07	0 41	0 26	0 018	0 014
10	DXI (34)	11 1	1 65	6 38	0 28	79 80	0 79	1 02					
11	Dudhsar	11 4	1 67	7 06	0 27	78 81	0 79	1 13	0 06	0 44	0 31	0 023	0 013
12	Badkalamkati	10 4	1 71	7 00	0 27	79 86	0 76	1 12					
13	Latisail	10 8	1 74	8 13	0 27	78 72	0 84	1 30	0 07	0 45	0 30	0 014	0 013
14	Bhasamanik	11 7	1 72	6 94	0 29	77 50	0 85	1 11					
15	Jhingasail	10 7	1 70	6 56	0 31	79 84	0 79	1 05	0 07	0 47	0 21	0 018	0 011
16	Nagra 08/6	10 2	1 71	7 00	0 28	79 50	0 81	1 12					
17	Khirajali	10 8	1 76	7 63	0 29	78 72	0 80	1 22	0 07	0 46	0 21	0 018	0 014
18	Chingrighusi	10 9	1 75	7 25	0 29	79 00	0 81	1 16					
	Averages	*10 9*	*1 64*	*7 12*	*0 27*	*79 12*	*0 79*	*1 14*	*0 068*	*0 45*	*0 26*	*0 018*	*0 013*

TABLE IV.

Showing the variation in chemical composition of parboiled and husked rices of Bengal (expressed as per cent on air-dry basis)

Variety	Moisture	Fat	Protein	Woody fibre	Soluble carbohydrate	Ash	Total nitrogen	Silica	P_2O_5	K_2O	CaO	Fe_2O_3
Aus												
1 Charnock	10 3	1 58	8 44	0 25	77 99	1 44	1 35	0 24	0 78	0 34	0 022	0 016
2 Dharial	10 2	1 53	7 88	0 17	78 83	1 39	1 26	0 19	0 61	0 32	0 021	0 015
3 Surjamukhi	9 5	1 65	7 00	0 24	80 40	1 21	1 12	0 15	0 77	0 36	0 016	0 014
4 Kumari	9 7	1 41	5 69	0 18	81 65	1 37	0 91					
5 PXS (8)	9 7	1 54	7 75	0 23	79 43	1 35	1 21	0 14	0 78	0 33	0 017	0 016
6 Bhutmuri	10 1	1 48	8 56	0 16	78 10	1 30	1 37	0 22	0 62	0 37	0 018	0 016
7 Jhanji	10 1	1 48	8 63	0 22	58 28	1 29	1 38					
8 Kataktara	9 9	1 58	6 75	0 21	80 28	1 28	1 08					
Averages	*9 94*	*1 53*	*7 69*	*0 21*	*79 41*	*1 33*	*1 21*	*0 19*	*0 71*	*0 34*	*0 019*	*0 015*
Aman												
9 Indrasail	10 1	1 31	7 67	0 15	79 59	1 18	1 23	0 08	0 55	0 42	0 067	0 015
10 DX1 (34)	9 4	1 93	7 00	0 28	80 37	1 02	1 12	0 08	0 58	0 44	0 070	0 015
11 Dudshar	9 3	2 00	8 00	0 26	79 25	1 19	1 28	0 06	0 53	0 45	0 064	0 014
12 Badkalamkati	9 4	2 20	7 56	0 26	79 21	1 07	1 21					
13 Latisail	9 1	2 21	9 06	0 27	78 23	1 13	1 45	0 08	0 64	0 41	0 034	0 014
14 Bhasamanik	9 7	2 12	7 88	0 27	78 90	1 13	1 26	0 07	0 62	0 43	0 066	0 015
15 Jhingasail	9 7	2 10	6 81	0 30	79 94	1 15	1 09					
16 Nagra 68/6	9 6	1 99	7 38	0 27	79 62	1 14	1 18					
17 Khirapali	9 4	2 35	8 56	0 28	78 16	1 25	1 37					
18 Chingrighusi	9 7	2 26	7 94	0 29	78 68	1 13	1 27					
Averages	*9 54*	*2 05*	*7 79*	*0 26*	*79 20*	*1 14*	*1 26*	*0 07*	*0 58*	*0 43*	*0 06*	*0 014*

TABLE V

Showing the average chemical composition of Aus and Aman paddies and differently treated Aus and Aman rices of Bengal (expressed as per cent on air-dry basis)

Sample	Class	Moisture	Fat	Protein	Woody fibre	Soluble carbo hydrate	Ash	Total nitrogen	Silica	P_2O_5	K_2O	CaO	Fe_2O_3
Paddy	Aus	10 10	1 15	6 23	9 87	66 47	6 18	0 99	5 20	0 64	0 31	0 039	0 016
Paddy	Aman	10 34	1 64	6 33	10 40	66 79	4 50	1 03	3 62	0 53	0 38	0 059	0 015
Sun-dried husked rice	Aus	10 30	1 58	7 18	0 59	79 17	1 37	1 14	0 20	0 76	0 33	0 021	0 015
Sun-dried husked rice	Aman	9 95	2 19	7 71	0 67	78 32	1 15	1 21	0 07	0 60	0 43	0 060	0 014
Sun-dried polished rice	Aus	11 2	1 04	6 76	0 22	79 98	0 83	1 08	0 18	0 45	0 19	0 017	0 013
Sun-dried polished rice	Aman	10 9	1 64	7 12	0 27	79 12	0 79	1 14	0 07	0 45	0 26	0 018	0 013
Parboiled husked rice	Aus	9 34	1 53	7 69	0 21	79 41	1 33	1 21	0 19	0 71	0 34	0 019	0 015
Parboiled husked rice	Aman	8 64	2 05	7 79	0 26	79 20	1 14	1 26	0 07	0 58	0 43	0 060	0 014

TABLE VI

Showing the variation in chemical composition of bran and husk having different treatments (expressed as per cent on air-dry basis)

Sample	Moisture	Fat	Protein	Woody fibre	Soluble carbohydrate	Ash	Total nitrogen	Silica	P_2O_5	K_2O	CaO	Fe_2O_3
(A) BRAN, SUN-DRIED												
Charnock	10 5	10 1	11 19	13 50	45 21	9 50	1 79	4 6	3 7	0 84	0 026	0 052
Jhanji	10 2	9 7	11 25	12 90	47 35	8 6	1 80	2 9	4 9	0 78	0 021	0 048
Averages	10 35	9 9	11 22	13 2	46 28	9 05	1 795	3 75	4 25	0 81	0 023	0 050
(B) BRAN, PARBOILED												
Charnock	9 9	9 7	11 31	13 4	46 49	9 2	1 81	4 7	3 3	0 84	0 025	0 051
Jhanji	9 7	9 2	12 68	13 1	46 82	8 5	2 03	2 7	4 6	0 82	0 022	0 047
Averages	9 8	9 45	11 99	13 25	46 65	8 85	1 92	3 7	3 95	0 83	0 023	0 049
(C) HUSK, SUN DRIED												
Charnock	9 5	0 82	3 50	40 1	27 08	10 0	0 56	17 6	0 49	0 31	0 025	0 092
Jhanji	9 3	0 78	2 62	39 5	29 50	18 3	0 42	17 5	0 22	0 22	0 021	0 095
Averages	9 4	0 80	3 06	39 8	28 60	18 6	0 49	17 55	0 35	0 26	0 023	0 093
(D) HUSK, PARBOILED												
Charnock	9 1	0 79	3 06	40 0	27 85	19 2	0 49	18 2	0 45	0 30	0 024	0 094
Jhanji	8 7	0 74	2 19	39 7	30 97	18 5	0 35	17 7	0 20	0 21	0 020	0 091
Averages	8 9	0 76	2 62	39 85	29 41	18 85	0 42	17 95	0 34	0 25	0 022	0 092

J, MR

TABLE VII

A comparison of the chemical constituents of paddies and rices of Bengal having different treatments with those of Bihar and Ceylon (expressed as per cent on air-dry basis)

Sample	Locality	Moisture	Fat	Protein	Woody fibre	Soluble carbo hydrate	Ash	Total nitrogen	Silica	P₂O₅	K₂O	CaO	Fe₂O₃	Authority
Paddy	Ceylon	12.01	1.91	,6.69	10.40	63.94	5.26	1.04						
	Bengal	10.22	1.39	6.28	10.13	66.63	5.34	1.01	4.41	0.544	0.34	0.078	0.015	J and K
Sun dried husked rice	Bihar	11.95	2.36	7.48	0.76	75.86	1.59	1.21						Sen (1916)
	Ceylon	11.21	2.47	9.26	0.47	75.69	0.90	1.48		0.58	0.36	0.049	0.014	J and K
	Bengal	10.12	1.83	7.44	0.63	78.74	1.26	1.19	0.13	0.70	0.38	0.052		
Sun dried polished rice	Bihar	10.89	0.88	7.25	0.20	79.99	0.79	1.16						Sen (1916)
	Ceylon	12.21	1.00	7.64	0.33	77.90	1.00	1.22		0.39	0.22	0.013		J and K
	Bengal	11.05	1.34	6.94	0.24	79.55	0.81	1.11	0.12	0.08	0.22	0.043		
Parboiled husked rice	Bengal	9.29	1.74	7.74	0.23	70.51	1.23	1.23	0.13	0.36	0.38	0.017		J and K
Bran, sun dried	Ceylon	12.51	10.12	14.05		38.72	1.71		2.93	0.43	0.28	0.039		
	Bengal	10.35	11.22	13.2		46.28	1.80		4.25	0.45		0.05		
Bran, par boiled	Ceylon	11.56	10.01	14.50		37.15	1.6	3.75	3.58	0.81	0.023	0.302		J and K
	Bengal	9.80	11.99	13.25		46.65	1.92	3.7	3.95	0.83	0.362	0.049		
Husk, sun dried	Ceylon	11.35		3.90	40.22	25.83	17.43	0.62	0.502	0.23	0.023	0.093		J and K
	Bengal	9.40		3.06	39.8	28.60	18.6	0.49	0.35		0.023	0.092		
Husk, par boiled	Ceylon	11.15		3.95	39.54	24.7	18.91	0.63	0.682	0.26	0.022			J and K
	Bengal	8.9		2.62	39.85	29.41	18.85	0.42	0.34	0.25				J and K

Note.—There is no analysis of Bihar paddy and parboiled rice. J and K indicate Joachim and Kandiah (1928)

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DIFFERENCE BETWEEN THE AUS AND AMAN PADDIES AND RICES OF BENGAL

Table V summarizes the average composition of Aus and Aman paddies and of rices of Bengal subjected to different treatments   Table V as also Tables II, III and IV make it clear that the Aman varieties of rice contain on the average more protein and fat than the Aus varieties For rice which contains a relatively excessive proportion of starch these constituents are very important from the nutritional standpoint and the analytical data obtained thus support the popular view that Aman rice is superior to the Aus varieties   The Aman rice also contains more calcium than the Aus   The ash content of the Aman rice is less than that of the Aus but Aman rice contains sufficient phosphoric acid although its phosphoric acid content is lower than that of the Aus rice   The Aman rice contains less moisture and silica but more potash than the Aus rice

Comparing the Aman and the Aus paddy one comes across the same relation as in the case of rice   The moisture content of the two varieties of paddy, however, appears to be identical

DIFFERENCE BETWEEN SUN-DRIED AND PARBOILED RICE

Tables V, II and IV bring out the difference between non-polished sun-dried and parboiled rices   The parboiled rices appear to contain slightly more protein than sun-dried rices of the same strains   Sun-dried rices appear to contain more moisture and woody fibre and a slightly higher percentage of fat than the parboiled variety   Working with six varieties of Ceylon rices Joachim and Kandiah (*loc cit*) found that except in the fat content which somewhat fell there was on the whole no appreciable change in the chemical composition as a result of parboiling

EFFECT OF POLISHING

It will appear from Table V and from a comparison of Tables II and III that polishing diminishes the fat, ash, phosphoric acid and calcium content of the rice Polishing brings down the phosphoric acid content by more than 25 per cent   It is clear that when these rices are polished in the mill, where polishing is more thoroughly done, the phosphoric acid content would fall dangerously low . Polishing lowers the calcium content more in the case of Aman than in the case of Aus   It also slightly lowers the protein content, on an average by 0 45 per cent Almost a similar lowering (0 2 per cent) has been observed by Sen (*loc cit*) but working with a single variety of Ceylon rice Joachim and Kandiah (*loc cit*) observe that polishing diminishes the protein content by about 1 per cent   The results obtained by the authors on the chemical compositions of polished Bengal rice agree in most respects with those of McCarrison and Norris (*loc cit*) on South Indian rices

Polishing increases the moisture content of Bengal rices by about 1 per cent Similar results have been obtained by Joachim and Kandiah (*loc cit*)   This can be explained by the fact that the moisture content of bran is less than that of the non-polished rice from which it is derived (*cf* Tables VI, IV and II)   The polished grain might also have a tendency to absorb moisture

COMPOSITION OF BRAN AND HUSK

Table VI (A and B) summarizes the composition of brans and (C and D) that of husks from two sun-dried and parboiled Aus varieties

Brans or the rejected residues obtained after polishing the rices would appear to be much richer in fat, phosphoric acid, potash and iron and to a lesser extent in protein than the rices from which they are derived   This points to a concentration of the fats, minerals and to a lesser extent of protein in the 'aleurone' layer of the grain   The bran contains much more fibre, ash and silica and less carbohydrate than the corresponding rice   The brans from the sun-dried and parboiled varieties appear to have almost the same composition

Husk appears to be poorer in fat, protein and phosphoric acid but considerably richer in woody fibre and ash—most of the latter consisting of silica   Husk is also richer in iron than the corresponding rice   Parboiling appears to have little effect on the composition of the husk

For the sake of comparison the composition of different varieties of paddies, rices and their by-products from Bengal, Bihar and Ceylon are given in Table VII

CONCLUSIONS

Chemical analyses were made of typical Bengal paddies, rices and other by-products and the changes in the chemical composition of the grain as a result of husking, polishing and parboiling studied   The following conclusions have been arrived at —

1   Different varieties of rice showed variation in their chemical composition

2   The husked rices are richer in protein, fat, carbohydrate and phosphoric acid than the corresponding paddies

3   Polishing results in a marked decrease in the fat, fibre, ash, phosphoric acid and potash content   The protein content decreases slightly but the moisture content increases on polishing

4   Parboiling affects the chemical composition of rice inappreciably, except the moisture and woody fibre contents which fall slightly

5   Bran, or the rejected residue after the polishing of rice, is richer in protein, fat and phosphoric acid content than the corresponding rice

6   Husk or the rejected residue after husking is highly deficient in protein and carbohydrate but richer in woody fibre, silica and ash

7   In general the Aman varieties are richer in protein, fat and potash contents than the Aus varieties

8   The Bengal rices are on the whole similar in chemical composition to the other Indian rices   They appeared, however, to be somewhat deficient in fat and protein contents

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BIOCHEMICAL INVESTIGATIONS ON DIFFERENT VARIETIES OF BENGAL RICE

Part II.

ENZYMIC DIGESTIBILITY OF RICE STARCH   ACTION OF TAKA DIASTASE

BY

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IN a previous communication Basu and Sarkar (1935) have reported on the chemical composition of different samples of Bengal rice Enzymic digestibility of the same varieties of rice is the subject-matter of the present investigation

It is quite probable that the readiness with which the carbohydrate and protein of the different varieties of rice would be digested and metabolized in the human body would be different with different varieties So far as the digestibility of the carbohydrate is concerned this appears all the more probable in the light of the investigations of Hammerstein (1908), Lang (1910), Nagao (1911), Reinitzer (1909), Pauletig (1917), Welzmuller (1921) and others on the action of amylases from various sources on starch

from different substances like potato, barley, maize, rice, etc These authors found that the extent of action of an amylase varied with the source of the starch In this connection mention must be made of an observation of Sherman and Baker (1916, 1919) who found that if the starch from different sources be first washed with very dilute alkali all these differences in the action of amylase vanish In the case of digestion of rice starch in the human body the starch is accompanied by proteins, inorganic salts and fats from the rice and differences in the digestibility of the starch from the different varieties are thus to be expected

In this paper the action of the Taka diastase amylase (Parke Davis & Co) on the starch of the different varieties of rice (both Aus and Aman) subjected to different treatments (for details *see* Part I of this paper, pp 745–758) and in presence of all its constituents, the difference in the digestibility of sun-dried and parboiled rice and also the effect of polishing the rice on the digestibility of starch have been studied

In order that the activity of the enzyme material Taka diastase (a preparation from *Aspergillus oryzæ*) might remain uniform, it was preserved in a refrigerator at 2°C to 3°C. Its action on pure starch was examined from time to time and found to remain constant

It is well known that due to inhibition by reaction products (Luers and Wasmund, 1922) the reaction velocity of this enzymatic reaction gradually falls off Readings were, therefore, limited to the first 10 minutes

Animal amylases have a higher optimum pH (pH 6 4 to 6 8) than amylases of plant origin (pH 4 6 to 5 2) The reactions in this paper were carried out at the optimum pH 4 92

EXPERIMENTAL

Rices having different treatments were powdered and sieved through a 150-mesh sieve and requisite quantity was taken and treated with a little cold water This was then placed in boiling water and boiled exactly for four minutes after which it was cooled and made up to known volume so that a four per cent suspension of starch was obtained A little toluene was also added to prevent bacterial action The substrate was prepared every day before the experiment

Each reaction mixture was made up of as follows 25 c c of starch suspension, 10 c c of phosphate buffer having a pH 4 92 were taken in a flask and kept in a constant temperature bath at 37°C ± 0 2° When the contents of the flask acquired the temperature of the bath, 2 c c of 0 2 per cent solution of Taka diastase were added thus making the total volume equal to 37 c c Five c c of the reaction mixture were withdrawn every 3, 6, and 10 minutes and the reducing sugar formed was estimated by the micromethod of Shaffer and Hartman (1921) Duplicate estimations were carried out in every case and the mean result is given in the following tables —

TABLE I

Showing the variation in the digestibility of different Aus varieties.

Variety	Time in mins	Amount of reducing sugar produced in mg		
		Sun dried polished	Sun dried non polished	Parboiled non polished
Charnock	3	4 73	4 69	5 22
	6	7 83	7 85	8 10
	10	12 01	11 0	11 36
Surjamukhi	3	5 51	5 45	5 79
	6	9 06	8 57	10 26
	10	13 36	12 06	13 63
PXS (8)	3	5 39	5 77	5 77
	6	8 77	8 77	9 00
	10	12 74	11 85	13 12
Jhanji	3	6 42	6 30	6 25
	6	9 86	9 66	9 81
	10	14 87	13 58	13 63
Kumari	3	5 34	5 17	5 99
	6	8 26	6 26	8 96
	10	12 67	12 10	14 16
Dhairal	3	6 31	6 36	6 36
	6	9 66	9 54	9 90
	10	13 88	14 06	14 16
Kataktara	3	5 45	5 45	5 66
	6	9 55	8 60	9 54
	10	13 26	12 10	13 05
Bhutmuri	3	6 43	5 99	5 71
	6	10 07	9 10	9 24
	10	14 10	12 43	13 32
Mean for 10 minutes		13 31	12 40	13 18

TABLE II

Showing the variation in the digestibility of different Aman varieties

Variety	Time in mins	AMOUNT OF REDUCING SUGAR PRODUCED IN MG		
		Polished sun dried	Non-polished sun dried	Non polished parboiled
Indrasail	3	5 07	4 88	5 99
	6	9 15	8 05	9 82
	10	13 09	11 69	12 67
Dudshar	3	3 68	4 60	6 19
	6	9 21	7 95	10 02
	10	13 09	11 53	13 95
Jhingasail	3	5 24	5 07	6 24
	6	8 68	8 26	10 82
	10	13 25	12 20	14 25
Latisail	3	4 69	4 41	5 71
	6	8 05	7 47	9 15
	10	11 45	10 95	12 01
Bhasamanik	3	4 69	4 50	5 24
	6	8 68	7 96	9 42
	10	11 85	11 03	12 48
Badkalamkati	3	4 29	4 22	4 97
	6	8 47	7 67	8 36
	10	11 85	10 62	11 44
Nagra	3	4 79	4 41	4 98
	6	8 47	8 15	8 42
	10	11 69	10 78	11 68
DXI (34)	3	4 60	4 41	5 01
	6	8 26	8 26	8 52
	10	11 69	10 78	11 68
Chingrighusi	3	4 51	3 75	5 51
	6	7 76	6 67	8 86
	10	11 85	10 12	12 67
Khiraijah	3	3 63	3 53	3 74
	6	6 82	6 67	6 82
	10	10 2	9 81	10 42
Mean for 10 minutes		11 97	10 95	12 33

DISCUSSION

Amongst the Aus varieties Charnock appears to be the least digestible Of the Aman varieties Khiraijah is the least and Jhingasail the most digestible

Both in the case of Aus and Aman varieties parboiled rice starch is more digestible than that from the sun-dried rice   This is to be expected in view of the fact that in the process of parboiling, rice starch is probably dextrinized to some extent

In case of both Aus and Aman, polished rice starch is more digestible than the starch from the corresponding non-polished rice   The bran which is rich in ether soluble substances, ash and proteins thus appears to contain some inhibitors for the action of Taka diastase amylase

Tables I and II indicate that towards Taka diastase the Aus varieties are more digestible than the Aman   People, however, believe that they can more easily digest Aman rice than the Aus   It must be noted that Taka diastase is not an amylase produced by the human body but by *Aspergillus oryzæ* Further investigations with amylase secreted by the human body as also investigations on the enzymic digestibility of the proteins of different varieties of rice are in progress

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THE CHEMISTRY AND PHARMACOLOGICAL ACTION OF *TODDALEA ACULEATA*

BY

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Toddalea aculeata (Hind   Janglikalimirchi , Tamil   Milakarnai) is a climbing shrub of the Rutaceæ order and is found in the lower Himalayas and in western and southern India   The whole plant, more especially the root-bark, is pungent and aromatic, and is used in Ayurvedic medicine for its tonic, stimulant and anti-pyretic properties , it is also reputed to be a specific for remittent fever   As a result of a few early trials with an infusion of the drug in the Madras Hospital, it was considered superior to quinine as an anti-pyretic and to calumba as a tonic, but recent investigations by Vyas and Bhatia (1932) have disproved its utility in malaria fever

A considerable amount of chemical work has been done on the drug, chiefly because of the celebrity it had attained at one time in European medicine under the name of 'Lopez root'   From the essential oil of the leaves have been isolated citronellal, linalool and a camphoracious substance, M P 96 5°C to 97°C (Hooper, 1893)   The bark has been found to contain resin, bitter stuff, tannin, citric acid, sugar, pectin, starch, etc , and to yield on incineration an ash rich in manganese (Schnitzer, 1862)   An alkali soluble glucoside, originally thought to be Hesperidine, had also been isolated, but Oersterle and Wander (1925), on investigating this more closely, have identified this as the glucoside Diosmin Perkin and Hummel (1895) examined the root-bark and isolated from it a yellow alkaloidal hydrochloride , they considered this to be berberine hydrochloride

from the result of a platinum determination of the platinic chloride and the general behaviour of the hydrochloride   The presence of no other alkaloid was detected by them   They also obtained a resin from which no crystalline material could be isolated

As the result of the investigations recorded here it has been found that berberine is totally absent in the bark, as shown by the test of Gordin which would be positive in the presence of even 0 02 per cent of berberine in the root-bark   On the other hand, it contains two alkaloids, one of which gives a yellow hydrochloride closely resembling berberine hydrochloride in appearance and having very nearly the same platinum content in the platinic chloride, and which could, therefore, be very easily mistaken for berberine   The following properties, however, served to distinguish it from berberine, parallel experiments having been undertaken with the two alkaloids This alkaloid which we have named *Toddaline* is a tertiary mono-acidic base having the formula, $C_{20}H_{21}NO_4$ It is colourless, melts at 269°C to 270°C when crystallized from chloroform, and is *completely* insoluble in hot water unlike either of the two modifications of berberine, and is nearly insoluble in absolute alcohol   The hydrochloride and the nitrate form yellow needles like the corresponding salts of berberine, but here again their sharp melting-points as well as the ease with which very dilute aqueous ammonia regenerates the alkaloid serve to distinguish it from berberine, the latter not being precipitable by very dilute ammonia   It contains two methoxyl groups like berberine, but differs from it in having one N-methyl group

The other alkaloid which has been named *Toddalinine* ($C_{19}H_{15}NO_4$) is a very strong base requiring liquor ammonia or sodium hydroxide to regenerate it from its salts   On this property depends its separation from toddaline   A complete description of the extraction and the purification of toddalinine and of a lactonic principle and an account of their properties have been published by two of us (Dey and Pillay, 1933)

EXPERIMENTAL

The root-bark examined was obtained through the Forest Department of Madras   The roots from two different localities were examined, the plants in each case being identified by the Botanical Department, Presidency College, Madras, but no difference in the products except in their relative amounts was detected

Absence of berberine in the root-bark —For the detection of berberine, both Gordin's (1901) method and the colour reactions with chlorine water were tried An alcoholic extract of 20 g of the root-bark was evaporated down, taken up with 20 c c of hot water, 10 c c of the aqueous filtrate made alkaline with 2 c c of 10 per cent sodium hydroxide, kept overnight to settle, filtered, warmed to 50°C and 5 c c of acetone added   There was no turbidity even on dilution and keeping overnight, while even one milligram of berberine under these conditions forms an appreciable precipitate   A similar extract made out of 1 g of the wood of *Coscinium fenestratum*, containing 3 per cent of berberine, gave a good yield of the crystalline acetone compound of berberine

Extraction of the root-bark —The following method was found to work most satisfactorily   The well-powdered root-bark was percolated six to eight times with methanol in the cold and the collected filtrate evaporated, first under ordinary and finally under reduced pressure

Separation of alkaloidal matter —The thick brown residue thus obtained was extracted first with hot water, then with hot 10 per cent acetic acid, cooling it each time before pouring off the extract in order to avoid too much of the resin going into solution To the collected filtrate was added an excess of strong potassium nitrate solution It was kept overnight and filtered The residue contained the nitrates of the alkaloids highly contaminated with resinous impurities, which were removed by grinding with small amounts of an equal mixture of methanol and ether, and filtering till the precipitate was pure yellow Small quantities of the nitrate of the weak base which went into solution were recovered later and added to the main portion of the alkaloid

Separation of the two alkaloids —The combined alkaloidal nitrates were ground with 10 per cent caustic soda solution, filtered, well washed with water and then dissolved in 10 per cent acetic acid when most of it went into solution The filtrate was collected, nearly neutralized with dilute ammonia, filtered from some resin which had separated and then the toddaline was precipitated by a slight excess of dilute ammonia Ten per cent caustic soda precipitated toddalinine from the filtrate

Characterization of toddaline and its salts —The crude toddaline was dissolved in one per cent acetic acid, nearly neutralized with dilute ammonia, kept overnight and filtered from some resinous impurities which had separated, and then precipitated by the addition of dilute hydrochloric acid The orange-yellow hydrochloride was crystallized twice out of hot water and the base reprecipitated by means of ammonia It was obtained as a white amorphous powder melting at 255°C to 258°C On slow evaporation of a chloroform solution it deposited small needles which were separated from a little impurity by washing with ethyl acetate and then melted at 269°C to 270°C The crude alkaloid seems to undergo a curious change into a white crystalline insoluble powder when it is rubbed with absolute alcohol, to which a drop of strong ammonia has been added This powder melts at 204°C to 206°C only, although it does not contain any alcohol or water of crystallization, and appears to be only another form of the alkaloid since it gives the same hydrochloride as the original alkaloid

(1) 23 89 mg (dried at 110°C) *in vacuo* 61 80 mg CO_2, 12 82 mg H_2O, C, 70 53 , H, 5 96
(2) 20 40 mg   ,,   ,,   52 60 mg CO_2, 10 77 mg H_2O, C, 70 33 , H, 5 87
(3) 22 71 mg   ,,   ,,   58 78 mg CO_2, 12 30 mg H_2O, C, 70 58 , H, 6 02
     8 729 mg   ,,   ,,   0 354 c c N_2 at 28°C and 762 mm N, 4 61
$C_{20}H_{21}O_4N$ requires C, 70 79 , H, 6 19 , N, 4 13 per cent

Toddaline is almost insoluble in the usual organic solvents except in chloroform but the rotation could not be found even in this medium because of the slight rosy fluorescence Nitrous acid has no action on it and so the base appears to be of a tertiary character

The molecular weight was found by the Rast method 9 4 mg (dried at 110°C *in vacuo*) in 82 2 mg camphor gave a depression of 13 1°C M W =349 1

$C_{20}H_{21}O_4N$ requires M W 339

Determination of methoxyl and methylimino groups, by Reynolds' method (Allen, 1929), showed the presence of two methoxyl and one methylimino groups 0 1476 g (dried at 110°C *in vacuo*) gave at 140°C 0 2082 g Ag I, OMe= 18 62 per cent

At 360°C, 0 1034 Ag I, NMe=8 65 per cent $C_{17}H_{12}O_2$ $(OMe)_2$ (NMe) requires OMe, 18 29, and NMe, 8 56 per cent

Toddaline hydrochloride —It can be crystallized from dilute acetic acid, hot water or hot alcohol from which it comes down in stout bright-yellow needles melting at 205°C to 206°C , and containing one molecule of water of crystallization, which is rapidly lost on heating at 110°C *in vacuo*   On prolonged heating the salt appears to undergo slow decomposition and turns brown, the base liberated with alkali being impure and coloured   It is fairly soluble in alcohol and hot water but quite insoluble in the presence of excess of the mineral acids   The rotation could not be determined owing to the intense colour of the hydrochloride

18 42 mg (air dried) gave 40 99 mg CO_2 9 88 mg H_2O C, 60 68 , H, 5 96 per cent
0 1042 g gave 0 0370 g of AgCl , Cl, 8 79 per cent
$C_{20}H_{21}O_4$ N HCl H_2O requires C, 60 99 , H, 6 10 , Cl, 9 01 per cent

Toddaline platini-chloride —The hydrochloride was dissolved in hot water, cooled, and a slight excess of a 7 per cent solution of chloroplatinic acid was added   A yellow oily precipitate formed immediately, which on standing overnight, crystallized spontaneously in long slender needles melting at 254°C to 256°C and containing no water of crystallization

(1) 56 30 mg gave 9 93 mg Pt	Pt, 17 64	per cent
(2) 56 32 mg gave 9 96 mg Pt	Pt, 17 68	,,
$(C_{20}H_{21}O_4N)_2$ H_2 Pt Cl_6 required	Pt, 17 94	,,

Toddaline auri-chloride prepared similarly, using a 10 per cent gold chloride solution, was obtained as an amorphous orange-yellow precipitate insoluble in water, alcohol or dilute hydrochloric acid and melting at 201°C to 202°C

82 85 mg (dried at 110°C) gave 23 95 mg Au   Au=28 91 per cent

$C_{20}H_{21}O_4$ N HAu Cl_4 requires Au 29 04 per cent

Toddaline nitrate was thrown down completely on adding excess of dilute nitric acid to a solution of the acetate   It crystallized from dilute acetic acid or hot alcohol in brilliant yellow needles softening at 235°C and melting at 239°C (decomp)

13 81 mg (dried *in vacuo* at 110°C) gave 0 855 c c N_2 at 31°C and 762 mm N, 6 97 per cent

$C_{20}H_{22}O_7N_2$ requires N, 6 97 per cent

Toddaline picrate —This was precipitated by adding a slight excess of aqueous picric acid to a solution of toddaline acetate, in brilliant needles, insoluble in boiling water or alcohol and melting at 237°C to 238°C

5 73 mg (dried at 110°C *in vacuo*) gave 0 525 c c N_2 at 32°C and 762 mm N, 10 21 per cent

$C_{20}H_{21}O_4$ N $C_6H(NO_2)_3$ OH requires N, 9 86 per cent

Toddaline sulphate —Precipitated from a solution of the acetate by dilute sulphuric acid, formed a yellow crystalline solid insoluble in hot alcohol and dissolving very sparingly in hot water   It crystallized from dilute acetic acid in yellow needles which darken from 210°C and melt with decomposition at 236°C to 239°C

Colour reactions of the alkaloids

Reagent	Toddalino	Toddalinine	Berberine
Concentrated H₂SO₄	Orange, quickly turning deep blood red On warming reddish brown On dilution cloudiness	Blood red turning dirty brown On dilution green with cloudiness	Orange yellow On warming olive green On dilution cloudiness
Concentrated HNO₃	Blood red, turning reddish brown On dilution orange	Reddish brown On dilution turbidity	Red turning reddish-brown On dilution orange
Frohde	Reddish brown, slowly brownish black	Dirty pink, turning dirty brown	Greenish brown, slowly turning brownish black
Erdmann	Orange, turning red On dilution colour discharged	Pink red, turning blood red On dilution greenish with turbidity	Deep orange, turning greenish brown On dilution turbidity
Sulpho-vanadic acid	Greenish brown	Deep red	Reddish-brown, turning violet

PHARMACOLOGICAL ACTION

The alkaloid, toddaline, is very irritant to the mucous membrane and the subcutaneous tissues A 2 5 per cent solution injected under the skin into rabbits produced much irritation and a sloughing open ulcer resulted in the course of a few days Subcutaneous injection of 100 mg per kilo of body-weight caused death in frogs and rats in a couple of hours

Because of its reputed anti-pyretic action we tested it on fevered rabbits according to Killiani's method Nine experiments were done, the doses ranging from 50 mg to 150 mg , the average weight of the rabbits being 1 7 kilo Either no fall in temperature occurred or the fall was no greater than that occurring in the control animals

Intravenous injections of as small a dose as 2 mg invariably causes a temporary arrest of respiration followed by spasmodic breathing This effect is seen in decerebrated animals as well, where the lung-volume also is appreciably diminished, and appears to be due to constriction of the bronchial muscle

Such small doses also markedly increase the secretion of saliva which dribbles down and forms a pool around the animal's head

Small doses (2 mg) cause a slight rise of carotid blood-pressure which disappears on repeated injections This initial rise is seen after vagal paralysis and in spinal animals, but is abolished after ergotoxin No appreciable effect is seen on the heart either *in situ* or isolated On perfusion of the frog's blood vessels *in situ* the outflow is markedly diminished pointing to vasoconstriction The volume of the spleen and the intestines is diminished This is due both to vascular constriction and contraction of the plain muscles, as the intestinal movements have been found to be

stimulated   A very marked but transient contraction of the urinary bladder also occurs

Experiments show that the lifting power of the skeletal muscle is greatly increased by the drug   In the case of the frog's gastrocnemius *in situ* this was increased three times   Direct application of a 5 per cent solution to the skeletal muscles causes a marked and sustained increase in tone

SUMMARY

From the root-bark of *Toddalea aculeata,* two alkaloids, *Toddaline* and *Toddalinine,* a lactonic non-nitrogenous principle, and a glucoside have been isolated   The alkaloids of which toddaline forms the major part have been shown to be different from berberine   Toddaline has been characterized by preparations of derivatives, such as nitrate, hydrochloride, platini-chloride, etc, and by well-marked colour reactions

The alkaloid has no anti-pyretic effect   It is irritant to the mucous membrane and subcutaneous tissues   It has no action on the heart but small doses raise the blood-pressure   Plain muscles of the bronchi, the blood vessels, the intestines, the spleen and the bladder are all stimulated to contraction   Skeletal muscle immersed in a 5 per cent solution shows a marked increase in tone   The load lifting power of skeletal muscle is increased   A marked increase in the salivary secretion also occurs

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ACTION OF EMETINE ON THE ACTIVITY OF THE ADRENAL AND THYROID GLANDS

BY

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CHOPRA and his co-workers (1928) showed that in Belgian hares after intravenous injections of organic compounds of antimony, there was a definite increase in the residual epinephrine content of the adrenal glands as compared with the normal gland   After the first two or three injections the increase was slight, but after a larger number of injections (ten) the increase was definite and marked, the adrenalin content being nearly doubled   It has also been shown that large doses of organic arsenicals produce a remarkable decrease in the chromaffine substance and of the residual adrenalin in the glands and that arsphenamine, in therapeutic doses, produces partial exhaustion of the adrenalin content

In this paper it is proposed to describe the effects produced by injections of emetine on the residual adrenalin content of the suprarenal glands and on the iodine content of the thyroid gland in experimental animals   Carl (1911) studied the effects of strychnine on the chromate reaction in frogs, and found it had a definite action on the suprarenal content of the chromaffine system   Bose (1931), in his experiments with the albino type of the Himalayan rabbits, showed that injections of emetine hydrochloride produced a marked intensification of insulin response and concluded that the effect is probably due to depression of the action of the suprarenals by the toxic effect of the drug on the chromaffine system   Clinically the possibility of producing symptoms of poisoning by the long continued administration of emetine in therapeutic doses is well recognized   Leiby (1930) reported a fatal

case of poisoning in an adult female due to the cumulative action of emetine after repeated therapeutic doses   In this patient death occurred from vasomotor collapse after a total dosage of 1 28 g , and post-mortem examination showed well-marked parenchymatous degeneration of the adrenals along with other anatomical changes   Acton (unpublished) found that the drug was useful in leucoderma and after a course of injections there was definite improvement in the leucodermic patches in many patients   This he considered was due to the depressant action of emetine on the adrenal glands

In view of these and other observations, and in view of the fact that emetine is largely employed in the treatment of amoebiasis, we carefully worked out the effect produced by emetine on these glands   In the series of experiments described Belgian hares were used   It has already been pointed out (Chopra *et al* , *loc cit*) that the results of estimation of the adrenalin content of the circulating blood are very variable   The best way to estimate the functional activity of the suprarenal glands appears to be to determine the residual adrenalin content of the glands after the administration of the alkaloid

EXPERIMENTAL

The Belgian hares were housed in different cages and were fed with green vegetables and gram, and thoroughly acclimatized for about 2 months before the experiment was started   They were all male animals having average weight ranging from 1 8 to 2 kilos   The dose of emetine was calculated on the basis of human dose, i e , at the rate of 1 mg per kilo of body-weight which is approximately the therapeutic dose given in human beings   Most of the animals, however, developed diarrhœa with hæmorrhage after such doses and the dose had to be reduced to half, and this was well borne   Each rabbit received eight successive daily injections, and on the ninth day the animals were killed   The adrenals were carefully dissected and removed and epinephrine was extracted by means of trichloracetic acid solution   The assay of the epinephrine content of this extract was carried out by Dale's modification of the biological method described by Elliot (1912) as well as by the colorimetric method   Rabbits Nos VII, VIII and IX were used as controls and kept under similar conditions

Biological assay —The injections of the extract of the glands were given into the femoral vein of decerebrated cats at equal intervals   The volume of the unknown solution containing adrenalin was kept constant and it was injected in a given time and washed through by a definite quantity of saline in all cases   The curves of carotid blood-pressure were recorded on a slowly moving drum and compared with those produced by a standard solution of adrenalin prepared by P D & Co   The reaction was considered identical if the curves coincided with each other both in their height and duration   The results obtained are given in column 5 of Table I

Colorimetric estimation of adrenalin —For details of this technique reference should be made to the previous paper on this subject   Certain modifications in the original procedure were, however, made   The estimations of adrenalin were carried out by using the protein-free trichloracetic acid extract as described by Baker and Marrian (1927) and Folin's (1913) technique as modified by

Barker, Eastland and Evers (1932) To 1 c c of this trichloracetic acid extract 0 25 c c of Folin's reagent was added followed by 0 75 c c of 5 per cent caustic soda solution The potassium persulphate test, as recommended by Barker, was not used as laboratory conditions did not allow of it The results are given in column 6 of Table I —

TABLE I

Belgian hare rabbit number	Weight before injection, g	Total emetine, mg	Weight after injection, g	EPINEPHRINE CONTENT PER GRAMME WEIGHT OF GLAND	
				Biological method, mg	Colorimetric method, mg
I	1,810	8 77	1,885	1 0	2 24
II	1,755	7 55	1,813	1 056	2 52
III	1,815	7 0	1,732	1 096	2 35
IV	1,630	6 76	1,625	0 87	1 85
V	1 707	7 0	1,785	0 95	2 00
VI	1,785	7 6	1,830	1 02	2 28
VII	2,080	Control	2,040	1 42	3 50
VIII	1,775	,,	1,800	1 44	3 64
IX	2,100	,.	2,190	1 36	3 42

DISCUSSION

The results given in the table show that the epinephrine content in normal rabbits, as estimated by the biological method, varied from 1 36 mg to 1 44 mg per gramme weight of the gland With the colorimetric method the results were higher and varied from 3 42 mg to 3 64 mg per gramme of the gland The epinephrine content of the adrenals after a series of eight injections of emetine as determined by the biological method is given in column 5 It will be seen that the epinephrine content is definitely lower, varying from 0 87 mg to 1 096 mg per

gramme weight of the gland   The results as determined by colorimetric method, given in column 6, show that, after a series of injections, the epinephrine content becomes less than that of the control animals and varies from 1 85 mg to 2 52 mg per gramme weight of the gland, as compared to 3 42 mg to 3 64 mg per gramme weight in the control animals   These figures though much higher than those obtained by the biological method were, however, sufficiently conclusive as regards the action of the emetine injection on the adrenal glands

EFFECTS ON THE IODINE CONTENT OF THE THYROID GLAND

The inter-relationship between the thyroid and suprarenal glands has been known for a long time   The iodine content of the thyroid gland was worked out simultaneously in order to establish any relationship after injections of emetine It is well known that after feeding with thyroid gland, and in pathological hyper-thyroidism, the adrenalin output is increased   The synergism of the thyroid and the phæchrome system is best evidenced by direct stimulation of the thyroid or adminis-tration of thyroid or iodine   This fact is well in keeping with the hyperplasia of the adrenal medulla (Pettavel, 1912 , Herring, 1915, 1920) which has been observed sometimes in Graves' disease   The increase in the functional activity of the thyroid gland is associated with increase in its iodine content, and this may be considered as a criterion of its functional activity after injections of emetine   The iodine content was estimated according to a modification of the technique described by Gorak (1926)

The glands are carefully freed from extraneous tissues, weighed and put in a small porcelain crucible with about 0 5 g of anhydrous sodium carbonate and about 0 3 g of finely powdered potassium nitrate , 0 5 c c of distilled water is then added and the crucible is gently heated over an asbestos board and the contents evaporated to dryness   The crucible is then heated to dull red-heat over a naked flame, till there is no dark part in the melt and until there is no evolution of gas   The melt while still warm is taken up with 5 c c of distilled water and transferred to a 150 c c Erlenmeyer flask, the crucible being thoroughly washed with about 20 c c more of distilled water and the washings added to the contents of the flask   A few small glass-beads are then added and the neck of the flask is washed with a little water Six drops of a cold saturated solution of potassium permanganate is added and mixed and the contents boiled for about 10 seconds   The flask is then removed from the flame and 5 c c of 25 per cent sulphuric acid slowly added, the flask being gently rotated during this process   After the addition of 1 c c to 2 c c the solution usually becomes clear and the rest can be used for washing the neck of the flask   The still hot flask is again put over the flame and saturated potassium permanganate solution diluted five times with water is added drop by drop till a permanent rose-red colour is obtained   After boiling for 10 to 15 seconds, 0 1 c c of N/10 $NaNO_2$ solution is added to the boiling fluid when it decolorizes almost instantaneously and the neck of the flask is washed with a little water After 25 to 30 seconds more boiling 1 c c of 10 per cent aqueous urea solution is added, and the neck of the flask washed again   During this process the flask should be kept over the flame and the boiling must not be interrupted   After boiling it for about 1 minute more, the flask should be taken away from the flame, cooled in running water and titrated with N/100 sodium thiosulphate after

the addition of about 2 c c to 3 c c of 10 per cent potassium iodide solution and a few drops of 1 per cent starch solution

TABLE II

Iodine content of thyroid

Rabbit number	Iodine content in mg per gramme of tissue	REMARKS
1	0 2692	
2	0 2900	
3	0 2425	
4	0 1820	
5	0 2085	
6	0 2465	
7	0 3070	Control
8	0 3840	,
9	0 3580	,,

Table II shows that in the normal animals the iodine content of thyroid varied between 0 30 mg and 0 38 mg per gramme of the gland   After a course of injections of emetine the iodine content of the gland is decreased and runs more or less parallel with the adrenalin content   This shows that simultaneously with decrease of the adrenalin content of the suprarenals after injections of emetine, the iodine content of the thyroid also tends to decrease

SUMMARY AND CONCLUSIONS

1   The experiments on Belgian hares show that after a series of eight intravenous injections of small (therapeutic) doses of emetine hydrochloride, there is a definite decrease in the residual adrenalin content of the gland as compared with the normal gland

2   The iodine content of the thyroid gland is decreased and runs more or less parallel with the adrenalin content

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AN IMPROVED METHOD OF RECORDING AMPLIFIED ELECTRICAL CHANGES IN TISSUES FOR PHARMACOLOGICAL WORK

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THERE are several existing methods for recording the rapid electrical changes in the tissues, e g, by the capillary electrometer, the string galvanometer and the two forms of oscillographs Matthews (1928) has given an excellent review of their uses and shortcomings The string galvanometer, in spite of its great value in recording the comparatively slow electric changes accompanying the heart beat, has many disadvantages In ordinary recording, the inertia of the string prevents it from giving an accurate record of the very rapid electrical changes such as those of the action currents of nerve Even in Einthoven's special type of string galvanometer for recording very rapid changes much loss of sensitivity is involved The instrument which Adrian (1926) used was a special type of capillary electrometer, in combination with a valve amplifier The sensitiveness of this system is great and the method of recording is convenient, but the records obtained with it are greatly distorted by the damping of the mercury, and a special machine is required for analysing them The illuminating arrangements for photographic recording in the string galvanometer and capillary electrometer are unsatisfactory as much loss of intensity results on account of the light having to pass through microscope objectives Rosenberg's moving loop oscillograph has been used for recording the nerve impulse but the records appear to be distorted, because when the applied potential changes rapidly, the loop over-swings and besides that instrument is not very sensitive There is no such objection in the case of the cathode ray oscillograph used by Gasser and

(777)

Erlanger (1922) as the moving system is a stream of electrons It has no appreciable mass nor damping, and there is *no chance of any of* its parts being damaged on account of excessive deflection Even this has certain drawbacks, the chief being that the stream of cathode rays has low actinic power and a single excursion will not affect a photographic plate, but that problem has now been partially solved by the introduction of a new type of Cossor tubes The moving iron oscillograph of Matthews is very good for general nerve work, but this also is not free from certain defects, which will be discussed later The modified oscillograph of Morelli (1932), which is also a sensitive apparatus, is a step forward but it does not always give an accurate response due to the rebound of the spring used in it The writers have recently effected improvement in the recording system and have developed a new instrument adapted for both physiological and pharmacological work This apparatus can accurately record the action potentials of nerves and muscles, and also that of a single nerve fibre The instrument is not readily liable to damage and its optical system is extremely simple Records can be obtained on plates or films with a high speed sensitivity Further, the sensitivity can be varied over a wide range by altering the number of valves employed

THE NEW APPARATUS

The principle of the system is to amplify the action potentials from the tissue by means of valve amplifiers and to allow this current to pass through the moving coil oscillograph The oscillations of the mirror in the oscillograph, that are produced by electrodynamic forces and are proportional to the impressed input current, will directly indicate the changes in the tissue

(a) *The moving coil oscillograph* —This instrument is designed on the following principle A coil of wire is wound on a light cylindrical ' former ' and the coil is suspended so that it can travel backwards and forwards in the direction of its axis through a distance of about 0 5 cm , but it is not capable of movement in any other direction It lies in the field of a permanent magnet as illustrated in Text-fig 1 (a) The coil when carrying current is thus acted on by an electrodynamic force, which sets it in motion at right angles to the plane of the current and the direction of the field When a current of varying intensity flows through the coil it undergoes a vibratory motion The coil is suspended by means of a special fibre ' spider ' which is fixed in the centre and which ensures only axial movements The chief advantage of the moving coil drive is the relatively large movement permitted by the free suspension The system is in fact identical with that of a moving coil diaphragm of a loud-speaker

The especially prepared strong permanent magnet has a field which is concentrated on the moving coil The section of the coil and its supports are shown in Text-fig 1(a), (b) and (c) It is built up of a light rigid non-conducting material and 90 turns of No 36 fine enamelled copper wire are wound round the coil From the rim of the coil a fine metal rod is attached at right angles to the cross section of the cylinder The axis of the vertical rod is connected to a small hinge joint The remoter end of this hinge is attached to another axis which carries a small concave mirror By this special arrangement the forward and backward movement of the first axis is converted into a transverse oscillatory

movement of the mirror and consequently an oscillatory movement is given to the reflected beam of light

The oscillograph is constructed in such a way that the frequency distortion is guarded by impedance matching of the output valves and the coil. The coil, however, possesses a certain amount of self-inductance, but that has been remedied by proper balancing of the circuit of the amplifier and also by adjusting the vibration of the materials used for fixing the coil which damps the over-swing. When a small potential is applied across the terminals of the coil [Text-fig 1 (b)], after a short interval an equilibrium is reached with a steady current flowing in the circuit. The coil carrying the current in the field of the permanent magnet is acted on by an electrodynamic force, proportional to the current. The movement of coil in its axial direction thus varies with the changes in the input potential. The moving system possesses mass owing to which there is a certain degree of damping. When there is no variation of current in the coil it is in a resting position, but as soon as the current strength alters the coil is moved to a new position by the difference
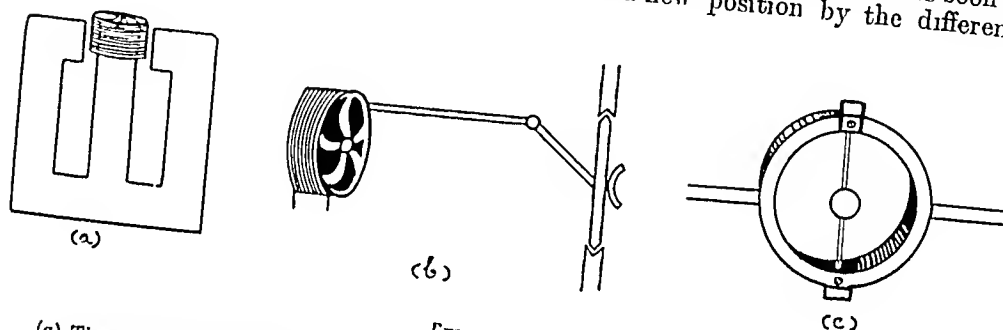
TEXT FIGURE 1

(a) The permanent magnet with the coil in position
(b) The oscillograph coil as attached to the vertical axis carrying the concave mirror (side view)
(c) The oscillograph mirror attached to the vertical axis (front view)

between forces. The resistance of air which the coil undergoes on account of rapid movement is almost negligible since the amplitude of vibration is small. When the frequency is very high, the air damping is quite sufficient to prevent over-swing. The fibre spider which fixes the coil responds to the rapid frequency change up to 30,000 per second. The current in the coil is proportional to the applied potential, and thus the displacement of coil is also proportional to the current. This moving coil has an advantage over the moving iron of Matthews' oscillograph in that the effect of hysteresis is avoided and the small residual effect is almost nil

(b) *The optical system and the photokymograph*.—A single filament lamp (4 volt type) is housed in a tube in front of which is attached a combination of lenses which throw a bright line of light on the concave oscillograph mirror. The reflected image is focused on the photokymograph. The vertical beam passes through a horizontal cylindrical lens and then through a narrow slit in front of the recording drum so that a point of light impinges on the photographic surface. The excursions of light to and fro are recorded on the

moving photographic film   The kymograph is worked by a special clock work constructed for the purpose, the speed of which can be varied according to the requirement (Text-fig 2)

(c) *The thermionic amplifier*—The amplifier designed to work with this oscillograph follows the most recent Q P P method   The amplifier is divided into two units   The first unit is R   C coupling, and the second is a 'B' class amplifier using ' B ' class valves designed to give a very large maximum output—an output much greater than obtainable from any single battery valve of class ' A ' type   The potentials to be measured are applied to the input of the first unit, which consists of three valves in a closed box   The output of the first unit is connected to the input of the second unit, in which there are amplifying valves followed by a driver valve coupled by a special 'B' class transformer and a ' B ' class valve   This valve is again coupled to the oscillograph coil by means of a matched transformer.
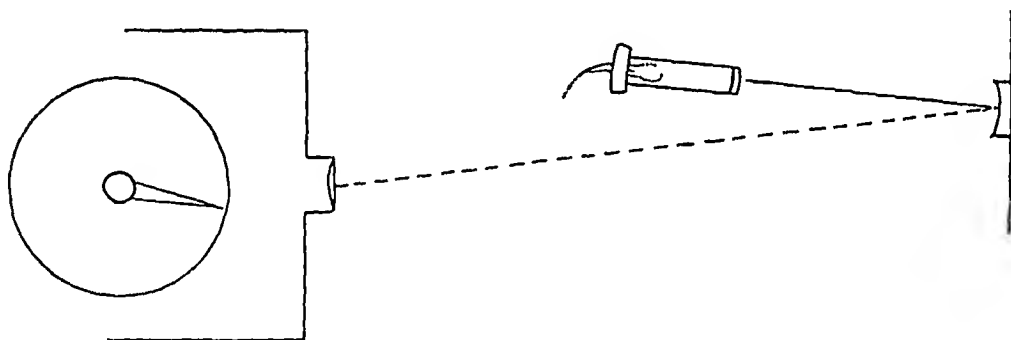
TEXT-FIGURE 2

Optical system with source of light and photokymograph

The standing current of a class ' B ' valve is very small, usually 3 milliamps, but more current is drawn as needed, when the signal input increases   Though the maximum undistorted output of class ' B ' valve is $1\frac{1}{4}$ watts, yet the mean anode current over a representative period of use is less than 8 milliamps which is sufficient to cause a deflection of 10 mm on the kymograph

The wiring of the amplifier is shown in Text-figs 3 and 4   The values of the resistances and condensers are also given   The components are of the best quality which are used for wireless sets   The first amplifier is contained without batteries in a case which is shielded by means of zinc sheets   The terminals of this unit are fixed to an ebonite panel   The valve holders are of anti-phonic type so that no outside mechanical disturbance is conducted through them   The shielding is connected to the earth   The second amplifier is not shielded, and the batteries are contained in the same wooden box where the components are fixed   The battery supply is different for the two amplifiers   The high tension batteries are accumulators in the first unit and dry in the second unit   In both the amplifiers, valves of two volt series are used   In the first unit a Cossor H L 210 is followed by a Mazda

H L 210 and by a Cossor P 220 , these three valves together give an amplification of approximately 5,616
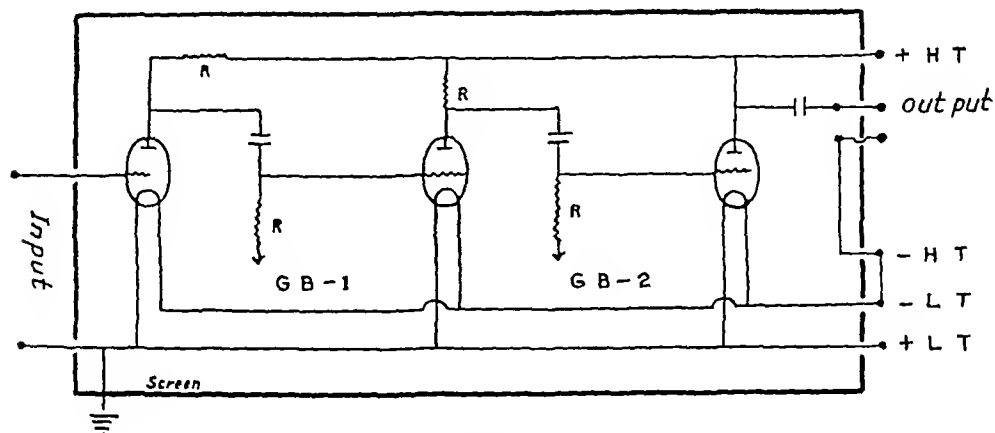
TEXT FIGURE 3

Circuit diagram of the first R C amplifier

In the second amplifier, as shown in Text-fig 4, the first valve is a metallized Mazda H L 2   The next one is a Driver P 220 Mazda and the last one coupled by B transformer is a P D 220 valve   Its amplification is 4,000   The total effective amplification is about 50,000
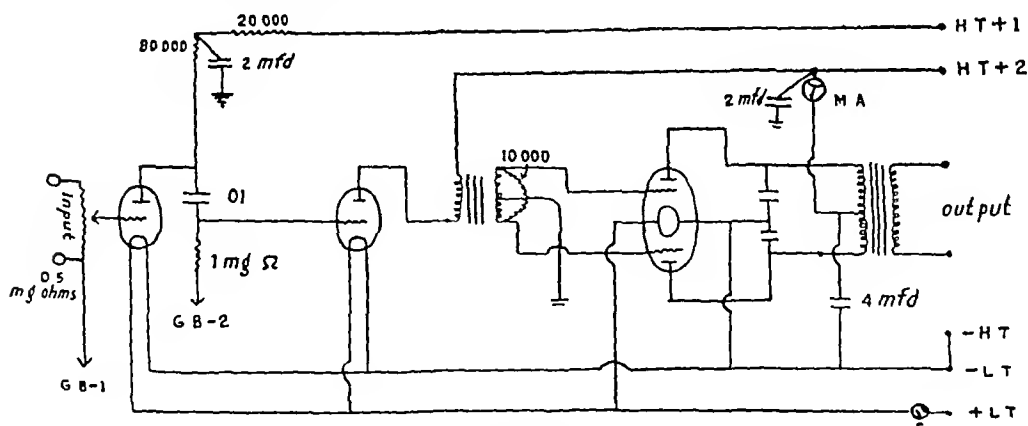
TEXT FIGURE 4

Circuit diagram of the ' B ' class second amplifier

As there may be various sources of distortion, the magnitudes of these should be determined for any amplifier before it can be used with confidence   By proper use of grid bias the straight line portion of the characteristic curve can be utilized

for working conditions   The inter-valve couplings may give rise to some trouble, but this can be overcome by careful balancing

ADVANTAGES

The construction of the apparatus is such that the oscillograph coil is suspended freely and thereby permits a large movement of the mirror   The fibre that suspends the coil responds faithfully up to the 'limits of audio-frequencies, namely 30,000 vibrations per second approximately   Moreover, the moving coil used in this apparatus has this special advantage over the moving iron used in various other instruments in this, that the effect of hysteresis is totally avoided and the residual effect is also negligible

(a)
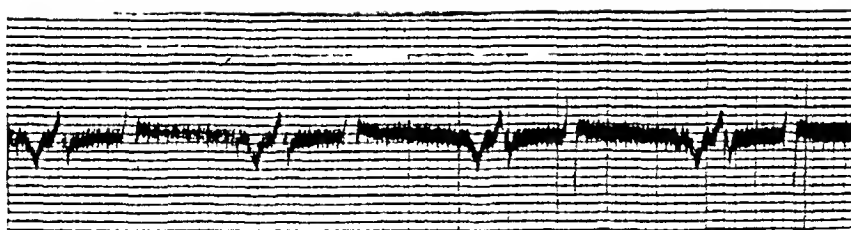
(b)

TEXT FIGURE 5

(*a*) Direct electrocardiogram of frog's heart at room temperature (25°C) with tho oscillograph

(*b*) Direct electrocardiogram of the same with the string galvano meter

The amplifiers also are constructed in such a way that it gives an undistorted output of 1 25 watt which drives the coil with a great force   Practically all the mechanical disadvantages that may ordinarily be present in an instrument of this type are overcome   By the proper balancing of the circuit by impedance matching of the valves and coupling devices the time lag is reduced to a minimum of the order of 0 0001 second   The instrument is moreover electrically unbreakable   If any large potential is accidentally applied to the input, the coil will move to a maximum position and cannot go further   Since the coil is transformer-coupled, and since

only the variation in the input is induced in it, no direct current flows through and thus there is no risk of the coil being burnt

*Nature of records due to action potentials in muscle and nerve —*The oscillograp records of frog's heart at room temperature are given in Text-fig 5 (a)   The leads were taken from the heart by means of electrodes of very soft silver tapes with platinized points   The tissues were placed in a metallic box, and leads were taken through an armoured wire to avoid external electric interferences

The electrocardiograms by the string galvanometer and by this new oscillograph, reproduced in Text-fig 5 (a) and (b), show that this instrument is as accurate as the string galvanometer and that the time lag due to an impressed potential change of heart-beat is less than that of the string galvanometer   Other usual features are accurately reproduced

When any impulse passes along the nerve fibre, its frequency can be recorded with this instrument as also its variation with the intensity of stimulus   Non-polarizable silver-silver chloride electrodes, prepared according to the method of Noyes and Ellis (1917), are used for experiments with nerves   If the muscle is stretched by means of loads of known quantity the frequency will increase with the increase of load (Text-fig 6)   If any drug acts on the nerve or muscle then the

TEXT FIGURE 6

Record of afferent impulses from a stretched muscle
at room temperature (25°C) and constant humidity

change of frequency in impulse can be recorded   The apparatus as designed can be utilized for studying the minute electrical changes induced by drugs or toxins in the nerve or muscle tissue   Whether the drug acts on the nerve itself or on the muscle fibre or in the central nervous system can be directly ascertained with the help of this instrument   The sensory and motor impulses can also be detected and recorded, in fact any small electrical change in tissues can be studied in detail

SUMMARY

The description of an improved type of oscillograph, electrically unbreakable, is given , the circuit of an up-to-date amplifier is also included   The adjusted valves give sufficient amplification for recording nerve impulse   Minute changes

of electric impulses induced by the action of drugs in nerves and muscles have been accurately recorded by this new apparatus

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CHEMICAL EXAMINATION OF THE BARK OF
MORINGA PTERYGOSPERMA

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Moringa pterygosperma belongs to the Natural Order Moringeæ It is a deciduous tree with small white or red flowers and cylindrical fruits varying in length from 6 inches to 2 feet The leaves, flowers and fruits are eaten as vegetables It grows wild in the sub-Himalayan tracts and is a very common plant almost in every part of India and Burma Its vernacular names are *Sahjna* in Hindi, *Sajina* in Bengali, *Murungai* in Tamil, *Da-tha-lwon* in Burmese and *Sobhanjana* in Sanskrit The two varieties of the tree are distinguished by the white and red colours of their flowers

Almost all parts of the plant, e g, root, bark, gum, leaf, flower, and seed, have been used for various ailments in indigenous medicine The detailed description of the botanical characters, distribution, medicinal uses and pharmacological action have appeared in a previous communication by Chopra *et al* (1932) The present paper deals with the results of the chemical examination carried out with the bark of the white-flowered variety of the plant A few mistakes which appeared in the previous paper in connection with the chemical investigation have also been corrected

Previous investigators only deal with the oil obtained from the seeds According to Cloez, the seeds contain 36 2 per cent of an oil known as Ben oil, having a specific gravity of 0 912 to 0 915, the oil does not turn rancid and is, therefore, much valued by watch makers as a lubricant The oil is also highly esteemed by

perfumers owing to its great power of absorbing and retaining even the most delicate odours   The tree grows extensively all over India and the seeds can be collected in enormous quantities during March and April   The seeds are rich in oil which can be easily expressed   By a little attention and a small outlay of capital India may easily and profitably supply the whole world with Ben or Moringa oil   The gum exuded from the bark may also find a suitable economic use

EXPERIMENTAL

The fresh bark when dried in the air lost about 72 per cent of its weight For a preliminary examination 50 grammes of the air-dried, powdered bark was successively extracted with petroleum ether (b p 35°C to 50°C), sulphuric ether, chloroform and absolute alcohol in a glass soxhlet   The extracts were freed from solvents, dried and weighed   Petroleum ether 0 71 per cent , sulphuric ether 6 47 per cent , chloroform 0 68 per cent and absolute alcohol 2 17 per cent   The chloroform and alcoholic extracts gave reactions for alkaloids

For a more systematic examination, 500 grammes of the drug were placed in a large extraction apparatus and extracted with the above solvents in succession The petroleum ether extract was found to contain some *fixed oil*, traces of an *essential oil* and a *phytosterol*   The sulphuric ether extract was found to contain some *organic acids* and a *waxy substance*   The chloroform extract gave distinct reactions of alkaloids   The alcoholic extract was found to contain *alkaloids,* some *organic acids* and a *resin*

An assay of the bark for alkaloids was carried out by different methods and the average of four analyses showed the presence of 0 105 per cent of total alkaloids The bark which was collected during the rainy season was found to contain as low as 0 025 per cent of total alkaloids

Essential oil —Five hundred and thirty-four grammes of fresh undried bark (equivalent to about 150 grammes of dried bark) were distilled in steam and the distillate extracted with ether   The yield of the essential oil was 0 026 gramme and it had a very pungent smell

Isolation of the total alkaloids —The dry coarsely powdered bark was thoroughly exhausted with rectified spirit (95 per cent alcohol) in a glass percolator   The alcohol was recovered by distillation, the last portion being removed under reduced pressure   The greenish-black semi-solid residue was extracted with one per cent aqueous hydrochloric acid   The acid aqueous extract was filtered, partially neutralized with caustic soda and allowed to settle   The clear supernatant liquid was filtered and extracted with petroleum ether to remove the oily impurities   It was then made alkaline with ammonia and extracted repeatedly with sulphuric ether The ethereal extracts were washed with water containing a little ammonia and then with distilled water   It was next dried with anhydrous sodium sulphate and the solvent removed   The residue was dark-brown and amorphous   The aqueous solution, which was exhaustively extracted with ether, still gave strong reactions for alkaloids   It was, therefore, extracted repeatedly with chloroform until the aqueous liquid gave only a faint reaction for alkaloids   The chloroform extract was washed, dried with anhydrous sodium sulphate and the solvent removed   The residue was dark-brown and amorphous

SEPARATION AND PURIFICATION OF THE BASES

The residue from the ethereal extract was dissolved in absolute alcohol and neutralized with alcoholic hydrochloric acid The solution was evaporated to dryness The residue was washed with petroleum ether, ether and benzene, which removed very little of the impurities The dry residue of the hydrochlorides was next boiled with anhydrous chloroform which removed most of the coloured substances leaving behind a colourless crystalline residue The amorphous alkaloidal residue from the original chloroform extract was also similarly treated The total hydrochlorides were thus divided into two fractions, one soluble in chloroform and the other not The hydrochloride of the chloroform-soluble base was, however, almost wholly soluble in chloroform The hydrochloride insoluble in chloroform was dissolved in absolute alcohol and the solution was allowed to evaporate slowly The crystals which separated were dissolved in a small quantity of absolute alcohol and precipitated with a mixture of chloroform and ether The substance was finally re-crystallized two or three times from absolute alcohol, until the melting-point remained constant In the previous communications it was reported as an alkaloid and provisionally designated as Moringine In view, however, of the more detailed chemical analysis and determination of the molecular weight and comparatively feeble pharmacological action the name may better be dropped while retaining the name Moringinine for the active alkaloid

For the isolation of a larger quantity of the bases we had three maunds of the dry bark extracted in a local factory The residue from the alcoholic extract weighed 3 lb 12 oz The yield of the bases was very much smaller (20 grammes) but a subsequent extraction in the laboratory gave about 21 grammes of the total hydrochloride and 13 grammes of the crystalline hydrochloride from 20 kilos of the bark

Properties and reactions of the hydrochloride and other derivatives of the isolated base —The hydrochloride crystallized in white glistening plates melting at 254 4°C It was freely soluble in water and alcohol but almost insoluble in ether and chloroform Its specific rotation $[a]_D^{30}$ was $+ 1 8°$ (C$=5$, in water)

It did not give any colour reactions with strong acids With Mayer's reagent it gives a white precipitate only in strong solutions With Kraut's reagent it gives an orange precipitate, with phosphomolybdic acid a lemon-yellow precipitate, with phosphotungstic acid a white precipitate and with alcoholic mercuric chloride colourless long flat needles

The *picrate* formed yellow woolly crystals insoluble in water but fairly soluble in alcohol, m p 195°C

The *aurichloride*, obtained from a concentrated aqueous solution of the hydrochloride with 10 per cent aqueous gold chloride, formed yellow hexagonal plates soluble in excess of water and freely soluble in alcohol, m p 170 8°C

The *platinic chloride*, obtained from a concentrated aqueous solution of the hydrochloride with 10 per cent aqueous platinic chloride, formed yellow rectangular prisms which are fairly soluble in water and freely soluble in alcohol, m p 221°C

The base isolated from the hydrochloride was a liquid

The elementary analysis of the crystalline hydrochloride gave the following results —

Carbon percentage found 58 96, 58 52, 58 73, 58 72 , average 58 74.
Hydrogen percentage found 7 43, 7 37, 7 22, 7 34 ,   „   7 34
Calculated for C_7H_9N HCl   C 58 53, H 6 97
Percentage of Cl in hydrochloride   24 71, 24 68
　　　　(Calculated for C_7H_9N HCl   24 73 per cent)
Percentage of N found   9 94, 9 94
　　　　(Calculated for C_7H_9N HCl   9 76)
Molecular weight obtained from analysis of the platinic chloride   106 8, 107 3
Molecular weight obtained from analysis of the gold chloride   111 9, 112 2
Molecular weight calculated for C_7H_9N HCl   107 0
The compound on heating decomposes and emits a smell of pyridine

From the various analyses and determinations of molecular weight, the compound appeared to be a simple base with a low molecular weight and is probably either a derivative of pyridine or one closely allied to the ephedrine group of bases It showed a feeble pharmacological action

Isolation and properties of the second active base, designated as Moringinine — The non-crystallizable hydrochloride which was separated from the total hydrochloride with boiling chloroform was allowed to crystallize after removal of the solvent As this did not crystallize even on long standing, it was further purified by dissolving in a small quantity of cold water, filtering and shaking successively with petroleum ether, benzene and ether None of these solvents could remove any impurities It was, therefore, made alkaline with caustic soda and the base extracted with chloroform The solvent was recovered and the base was obtained as a light-brown liquid No crystals separated from the liquid even on long standing in vacuum Attempts were made to crystallize the salts with hydrochloric, nitric, sulphuric, phosphoric and tartaric acids but none crystallized The salts gave strong reactions of an alkaloid with almost all the common alkaloidal reagents Picric acid, platinic chloride and auric chloride produced yellow amorphous precipitates

As the quantities of the second base were very small no further chemical investigation could be carried out It showed strong pharmacological action even several months after its preparation

SUMMARY

Two bases were isolated from the bark, the total amounting to about 0 1 per cent The method of isolation and separation is described in detail The one whose hydrochloride crystallized out has a simple structure and a low molecular weight The properties of some of the derivatives are described It had only a feeble pharmacological activity The base whose salts could not be crystallized had a strong pharmacological action resembling that of adrenaline Besides the bases, there were traces of essential oil, fixed oil, phytosterol, waxes, resins and organic acids

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THE VITAMIN C VALUE OF SOME COMMON INDIAN FRUITS, VEGETABLES AND PULSES BY THE CHEMICAL METHOD

BY

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In a previous paper (Ahmad, 1935) the chemical method for the estimation of vitamin C according to the technique of Tillmans as improved by Harris and his colleagues was studied, and it was shown that the method is of practical value for the assay of vitamin C in vegetable foods   Indeed it cannot be claimed that the method is specific, as in some tissues unknown reducing substances may be encountered which may interfere with the chemical estimation   But now substantial evidence is available that the values obtained by the chemical method agree generally with those obtained through biological tests

The chemical method has simplified very much the assay of vitamin C in natural foods   The biological method is long and tedious and does not give strictly quantitative values in the sense that the results cannot be expressed in numerical form   The values may vary according to the basal diet employed and the length of the test, and unless a large range of doses is tested the results are merely qualitative

In this paper the vitamin C values of some of the common Indian fruits, vegetables and pulses as determined by the chemical method are described and further evidence is presented that these values agree with their reputed antiscorbutic values within practical limits

EXPERIMENTAL

The vegetables examined were usually the locally produced varieties   They were purchased from the market and an attempt was made to obtain them in a fresh, young and healthy sound condition   When more than one variety is

available, the results refer to the more common and popular variety though often more than one variety was examined   The fruits available in the Calcutta markets come from all over India and are even imported from abroad   Usually the Indian-grown fruit was examined and care was taken to obtain samples from fresh healthy lots   In some cases, no doubt, the freshness of the sample could not be ascertained

Ten grammes of the vegetable tissue were thoroughly macerated and ground up with pure sand and 6 25 c c of 20 per cent trichloracetic acid   The extract was made usually in the cold, except in some kinds, for example cabbage, where it was not possible to extract the full amount of vitamin by cold extraction   The extracts were filtered through several layers of muslin and made up to 25 c c with water, and titrated against a standard solution of the indicator with the minimum loss of time

The standard solutions of the indicator were made by dissolving 20 mg of 2   6 dichlorophenolindophenol in 10 c c of water   The solutions were standardized against a standard solution of pure ascorbic acid which had been previously standardized with 0 01 N iodine   The solutions of the indicator were made fresh twice a week and stored in the refrigerator

In all cases the extracts were titrated against a known volume of the indicator, usually 0 1 c c to 1 0 c c depending upon the potency of the extract   The results are calculated as mg of ascorbic acid per 100 g of the vegetable

The results are shown in the following Table   The first column shows the common name of the fruit or vegetable which was examined, the second the ascorbic acid value in mg per 100 g as estimated by the chemical method   In the third column are given the reputed anti-scorbutic values of the fruit or vegetable as determined by the biological technique   These are also expressed in mg per 100 g vegetable   In the last column is shown the reference to the author from whose publication the biological value is taken —

The calculations of the results of biological tests in terms of ascorbic acid have been made on the basis that the average minimum dose of ascorbic acid to protect a guinea-pig weighing 300 g to 600 g over a ninety-days' period is 0 75 mg per day   Investigators who have attempted to ascertain this minimum dose have given values ranging from 0 5 mg to 1 0 mg per day   A careful and critical perusal of the literature on this subject, however, shows that 0 5 mg per day is too low and that 1 0 mg per day is too high   It appears that the average figure 0 75 mg per day represents a better approximation to the true value

It may appear unjustifiable to express the results of biological tests in the form of numerical value, and especially to place the results of tests carried out as early as 1912 when the problems of nutrition with regard to the accessory food factors were not quite so well understood, side by side with those obtained with the help of modern technique   As a matter of fact the biological technique of vitamin C estimation has undergone very little change, as a result of our increasing knowledge, since the days of 1912 when Holst and Frolich (1912) first produced experimental scurvy in guinea-pigs by feeding them on bran and rolled oats

The vitamin C value of vegetable foods

Columns — 1		2	3		4
Number	Name of food stuff	Ascorbic acid as estimated chemically, mg per 100 g	Ascorbic acid calculated from the reputed anti scorbutic value, mg per 100 g		Reference to the biological value as in Column 3
I	VEGETABLES —				
1	Potato	12 50	Cooked	3 75	Holst and Frolich (1912)
			Cooked	3 75	Chick and Rhodes (1918)
			Raw	7 50	Givens and McClugage (1920)
2	Celery	2 00			
3	Onions— young and fresh	10 50			
	stored	trace			
4	Garlic— stored	5 2			
5	Carrots	0 6	Cooked	3 0	Hess and Unger (1919)
6	Raddish— root	40 0			
	young leaves	113 8			
7	Turnip	28 0			
8	Cabbage leaves	40 5	Leaves	37 5-50 0	Delf (1918)
			,,	37 5-50 0	Delf and Skelton (1918)
9	Lettuce	<0 5			
10	Spinach	31 5			
11	Tomato— juice	25 9	Juice	25 0	Sherman, La Mer and Campbell (1921, 1922)
			Juice	19 0	Hess and Unger (1918)
			Whole	18 75	House, Nelson and Haber (1929)
			Juice, English	37 5	Delf (1924)
			Juice, S Afr	18 75	Delf (1924)

*The vitamin C value of vegetable foods—*contd

Columns — 1		2	3	4
Number	Name of food stuff	Ascorbic acid as estimated chemically, mg per 100 g	Ascorbic acid calculated from the reputed anti scorbutic value, mg per 100 g	Reference to the biological value as in Column 3
I	VEGETABLES—*contd*			
12	Cucumber	6 9	7 5	Embrey (1923)
13	Pumpkin	1 1	7 5	Wats and White (1931)
				Wats (1929)
14	Brinjal	22 5		.
15	Cauliflower	5 9	.	
16	Lady's Finger (Bhindi)	17 0		.
17	Patol .	13 1	.	
18	Karelā	51 0		.
19	Tôri ..	12 3	.	
20	Red pepper— fresh, green	45 5	83 3	Quinn, Burtis and Milner (1927)
	a local variety	34 0	Juice of Hun- 200 0 garian Paprika	Svirbely and Szent-Györgvi (1933)
	another variety	11 7		
	large, hilly	55 6		
21	Soya leaves	33 3		
22	Dhanya leaves	34 7		
23	Mint leaves	Deficient		
24	Indian corn— young cobs	8 05		..
25	Peas— fresh, green	7 25		
26	French beans— fresh, green	2 6		
27	Squash	2 5		.

The vitamin C value of vegetable foods—contd

Columns — 1		2	3	4
Number	Name of food stuff	Ascorbic acid as estimated chemically, mg per 100 g	Ascorbic acid calculated from the reputed anti scorbutic value, mg per 100 g	Reference to the biological value as in Column 3
I	**VEGETABLES**—*concld*			
28	Ginger—root	6 0		
29	Vegetable marrow	17 8	5 0	Wats (1929)
30	Mango—green	3 2		
II	**FRUITS** —			
31	Orange—juice	31 2	Juice > 25 0	Chick and Rhodes (1918)
	(a medium size orange 166 c o juice 50 2 mg ascorbic acid)		,, 50 0	Delf (1925)
			,, > 25 0	Eddy (1929)
			,, 50 0	Davey (1921)
			,, Jaffa 50 0	Bracewell and Zilva (1931)
			,, Indian 7 5	Wats and White (1931)
32	Orange—peel	1 2		
33	Tangerine—Nagpur	31 5		
34	Lemon—juice	38 5	Juice 50 0	Chick, Hume and Skelton (1918)
	(a medium size lemon 5 7 mg ascorbic acid)		,, 50 0	Davey (1921)
			,, 50 0	Delf (1925)
35	Lime—juice, fresh	16 8	Juice 15 0	Davey (1921)
			,, 15 0	Chick, Hume and Skelton (1918)
			,, 15 0	Wats and White (1931)

The vitamin C value of vegetable foods—contd

Columns — 1		2	3	4
Number	Name of food stuff	Ascorbic acid as estimated chemically, mg per 100 g	Ascorbic acid calculated from the reputed anti scorbutic value, mg per 100 g	Reference to the biological value as in Column 3
II FRUITS—*contd*				
36	Sweet lime— juice	31 2	Juice <15 0	Wats and White (1931)
	a Bombay variety	58 8		
37	Chakotra— pulp	56 8		
	juice	71·4		
38	Grape fruit— juice	31 2	B Honduras 50 0 and S Africa	Bracewell and Zilva (1931)
	(one fruit 215 c c juice 67 08 mg ascorbic acid)			
39	Mango— ripe fruit pulp	13 0	7 5	Wats and Eyles (1932)
	another graft .	15 3	.	
40	Papaya .	48 1	>15 0	Miller (1926)
41	Pine apple	28 5		
42	Leechi— Bengal	48 0		
	Mozaffarpur	25 0		
	Bengal leechis after storing at 0°C for 35 days	44 0		
	(9 5 leechis give 100 g pulp)			
43	Apple	0 1	<2 5	Holst and Frölich (1912)
			Juice 3 1	Robinson (1919)
	Kashmir	2 5	„ 6 8	Givens, McClugage and van Horne (1922)
			Bramley's seedling 25 0	Bracewell, Hoyle and Zilva (1930a)

The vitamin C value of vegetable foods—contd

Columns —	1	2	3	4
Number	Name of food stuff	Ascorbic acid as estimated chemically, mg per 100 g	Ascorbic acid calculated from the reputed anti scorbutic value, mg per 100 g	Reference to the biological value as in Column 3
II Fruits—*concld*			Other varie ties 3 75–15 0	Bracewell, Hoyle and Zilva (1930*b*)
				Bracewell, Kidd, West and Zilva (1931)
44	Guavas— pulp	90 0	>7 5	Embrey (1923)
45	Banana pulp— yellow	<1 0	Pulp 7 5	Eddy (1929)
			,, 5–7 5	Lewis (1919)
	red	1 4	,, 7 5	Givens, McClugage and van Horne (1922)
	green	2 1	Indian 7 5	Jansen and Donath (1925)
			Yellow 15 0	Eddy and Kellog (1927)
			Ind yellow 15 0	Wats and White (1931)
			Red 7 5	Wats and Eyles (1932)
			Green <5 0	Wats and Eyles (1932)
			Yellow 3 0	Embrey (1923)
46	Grapes	<1 0	<3 5	Chick and Rhodes (1918)
47	Melon	2 0–12 0		
48	Water melon	<1 0		
49	Pears	<1 0		
50	Peaches	1 0		
51	Plums (alucha)— yellow orange	<0 5		
52	Greengages	<0 5		
53	Pomegranate— juice (a medium size pomegranate 97 c c juice 15 1 mg ascorbic acid)	15 6	Juice 15 0	Sherman (1929)

The vitamin C value of vegetable foods—contd

Columns — 1		2	3		4
Number	Name of food-stuff	Ascorbic acid as estimated chemically, mg per 100 g	Ascorbic acid calculated from the reputed anti-scorbutic value, mg per 100 g		Reference to the biological value as in Column 3
II	FRUITS—*concld*				
54	Bael	7 6			
55	Jaman (20 jamans give 100 g pulp)	8 3			
56	Coco nut, green— pulp	3 4			
	Water from fresh— green	1 66			
	Water from ripe (water in one green co co nut = 224 o o — 3 72 mg of ascorbic acid)	5 0			
57	Chico	2 5	Palp	7 5	Embrey (1923)
III	LEGUMES AND DALS —				
58	Mung, green— dry seeds	3 0			
	sprouted	23 0	Sprouted	15 0	Santos (1921)
			„	25 0	Wats and Eyles (1932)
					Wats and Wodehouse (1934)
59	Mung, black— dry seeds	2 7			
	sprouted	10 5	Sprouted	18 75	Wats and Eyles (1932)
60	Mung, brown— dry seeds	2 3			
	sprouted	11 7			
61	Gram— white (Kabuli) dry seeds	3 0			
	sprouted	7 3			

The vitamin C value of vegetable foods—concld

Columns —	1	2	3	4
Number	Name of food stuff	Ascorbic acid as estimated chemically, mg per 100 g	Ascorbic acid calculated from the reputed anti scorbutic value, mg per 100 g	Reference to the biological value as in Column 3
LEGUMES AND DALS—*concld*				
62	Gram, brown— dry seeds	2 5		
	sprouted	7 8		
63	Lentils (Masur)— dry seeds	3 0		
	sprouted	15 0	Sprouted 15 0	Chick and Delf (1919)
64	Peas— dry seeds	2 7		
	sprouted	9 1	Germinated 15 0	Chick and Delf (1919)
65	Beans— dry seeds	1 25		
	sprouted	14 2		

CONCLUSIONS

A striking agreement between the values obtained by the chemical method and those of biological tests becomes apparent even from a superficial examination of the Table   Such variations that appear are natural, as fruits and vegetables must indeed be expected to vary considerably in their content of the anti-scorbutic vitamin according to their freshness and age   Since the biological test depends upon feeding a vegetable over a period of 2 to 3 months, there are obvious difficulties in obtaining that vegetable in an equal state of freshness from day to day for feeding purposes The biological test itself cannot possibly detect small differences in value   Several of the investigators quoted have not examined a sufficient range of doses to justify the results of their studies being expressed in numerical form   In most of these cases if a lower dose had been tested it might possibly have been found to be active Thus, wherever a critical examination shows that a sufficient number of doses was not tested, and other evidence is available in favour of a value varying rather widely from that quoted, the mathematical sign ' > ' (greater than) has been put against that figure to denote this fact   Taking these facts into consideration, the agreement between the values obtained by the two methods is indeed very close   Further support is thus available for the view that the chemical method is of practical value for the vitamin C assay of food-stuffs

Among the interesting facts that emerge from a study of the Table are the unexpectedly high anti-scorbutic values of some of the Indian fruits and vegetables Raddish leaves, largely eaten by the poor population of India, are exceedingly rich Karelā, another favourite vegetable, is a rich source Certain varieties of red pepper, brinjals, soya and dhanya leaves show high values On the other hand examples of poor vegetables may be found in the pumpkin and the cauliflower

Among the fruits the citrous family in India might appear to be somewhat poorer than its Western varieties Indeed such an impression has been created through some of the studies on Indian fruits (Harris, 1933) We have examined a large number of Indian oranges, tangerines, limes and sweet limes Their vitamin C values ranged from 30 mg to 40 mg of ascorbic acid per 100 c c of juice For the sake of comparison, we, at the same time, examined a number of imported oranges, namely, Jaffa, Californian ' Sunkist ', and the Australian But no higher values were obtained even for those We ascribe these rather low values to the conditions of storing in the moist and hot climate of Calcutta, rather than to the soil and the environmental conditions which would not affect the vitamin C content to the same extent as the observations of some of the investigators in this field might appear to suggest

Some of the Indian fruits which have been found to show unsuspectingly high values for vitamin C are the papaya, pine-apple, guavas and leechi Of these, the first-named three are cheap fruits and within the reach of the poorer classes Apples, bananas, melons, pears, peaches, plums, grapes, greengages, and chico have appeared to be rather poor

The study of legumes and dals confirms the well-known fact that they are poor in the dry condition but develop the vitamin on germination Green mung appears to develop the greatest amount of anti-scorbutic vitamin after sprouting Lentils, beans also show a greater increase in their value than gram, peas, masur and other pulses, though all of them showed appreciable increase

It appears that the anti-scorbutic vitamin tends to diminish on allowing the germination to proceed for a long time The highest figure for ascorbic acid seemed to reach even after 24 hours' germination under the climatic conditions of the Calcutta rainy weather Forty-eight hours later the ascorbic acid was always found to be rather less and decreased rapidly after that No explanation can be offered for this observation at the present stage of investigation

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ON THE NATURE OF PULMONARY TUBERCULOSIS IN ADULT PUNJABIS

BY

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THIS paper is mainly concerned with the relatively high degree of allergy to pulmonary tuberculous infection shown by adult indigenous inhabitants of the Punjab and with the fact that many such adult cases present features characteristic of the disease in children in Western countries   The observations were made in the Mayo Hospital, Lahore, an institution containing over 400 beds, of which 20 are reserved for adult cases of pulmonary tuberculosis   Tuberculous patients, like others, come from all parts of the province and sometimes from outside it, and are usually admitted in a more or less advanced stage of the disease   In all admitted cases the sputum contains tubercle bacilli   As post-mortem examinations are rarely obtained these observations are necessarily incomplete but they are founded on patients who remained a sufficiently long time in hospital to enable an estimate to be made of the rate of progress of the disease and of the nature of the disease process

Although tubercle bacilli may on occasions reach the lungs and tracheo-bronchial glands via the lymphatics or the blood stream, it is now generally agreed that fibro-caseous pulmonary tuberculosis results chiefly from air-borne infection Intra-thoracic disease has been experimentally produced in animals by way of the lymph stream and alimentary canal but, as Parsons (1934) points out, much larger doses of bacilli are necessary for infection by these routes than by the air passages Ghon (1912) showed that primary infection of the lungs in children gives rise to a proliferative lesion which may be so small as to be overlooked or as large as a greengage plum   From this lesion micro-organisms travel along the lymphatics to the regional lymph glands which become inflamed and may undergo caseation Such a sequence of events is sometimes referred to as ' Ranke's primary complex ' Enlarged tuberculous tracheo-bronchial and mediastinal glands may be found

post-mortem in patients who showed no evidence of pulmonary tuberculosis during life and no significant pulmonary lesions at autopsy, the initial focus having produced no symptoms and left little trace behind   Ghon's primary tubercle, as it is called, is usually situated at the periphery of the lung close to the pleural surface   When the intra-thoracic glands become involved there may be fever and other indications of toxæmia   Recovery from this condition usually occurs but if the infecting dose of bacilli is large or if the patient's resistance is low (as may be the case after a debilitating disease like whooping cough), instead of healing a rapid spread, or even generalization, of the infection may take place   Except when this happens a high degree of allergy develops in a few weeks and the tuberculin tests become positive

If in the allergic condition the lungs become infected the lesions are surrounded by an inflammatory reaction (peri-focal pneumonia) which, although harmful to the tissues, tends to destroy the invading organisms   Exudative phenomena characteristic of the ' secondary tuberculous state ' also sometimes occur   Exudations may take place into the lung tissue giving rise to what is called epituberculosis, into the pleural cavity or between the lobes of a lung   Other manifestations of allergy are phlyctenular conjunctivitis and erythema nodosum   When a person who has passed through this stage develops pulmonary disease in later life as a result of exogenous or endogenous re-infection there is an early tendency to localization by fibrous tissue, unless the infection is a massive one from erosion of a gland into a bronchus or from widespread dissemination through the blood stream   Localization indicates the development of immunity (the tertiary tuberculous state) and the localized disease is referred to as the ' adult ' type of pulmonary tuberculosis

Although allergy and immunity are often present together, both being produced by the union of antigen and anti-body (Topley, 1933), Rich (1933) has shown that they are not interdependent and that it is possible to separate them   His work indicates that the ' necrotizing inflammatory type of hypersensitiveness ' which is called allergy, far from being a mechanism of immunity, is a harmful process   In the immune non-allergic animal infection is overcome with a small amount of tissue reaction   Rich states that ' it is especially noteworthy of comment that in all of our various experiments in which allergy has been excluded the most striking characteristic of acquired immunity has always been the remarkable indifference of the tissues to the infecting bacteria which are undergoing destruction   This is exactly the state of affairs which obtains in natural immunity '   Sabin *et al* (1933) showed that rabbits rendered allergic to tuberculin by treatment with large doses of purified tuberculo-protein develop no immunity to infection with virulent tubercle bacilli   On the contrary, the duration of life after infection was shorter in such animals than in controls and the disease was more extensive and caseation and necrosis more intense

The reaction of the lung, therefore, to tubercular infection depends not only on the size of the infecting dose and the natural resistance of the host but on the reactivity of the tissues, that is, on the relative development of allergy and immunity   In Western countries the allergic state is chiefly associated with tuberculosis in childhood, while the immunity reaction is mainly seen in adults   Wood (1933) has represented diagrammatically in the following

manner the probable course of events following primary infection of the lungs in childhood —

lymph stream
Primary focus ⎯⎯⎯⎯⟶ Hilar glands × Arrest

lymph stream
Primary focus ⎯⎯⎯⎯⟶ Hilar glands ⟨ → Lethal invasion of the blood
→ Acute miliary tuberculosis. Death
→ Disseminated exudative lesions by blood stream × Arrest

lymph stream
Primary focus ⎯⎯⎯⎯⟶ Hilar glands × Arrest Re infection (exogenous or endogenous) ⟶ Local disease of lungs (phthisis)
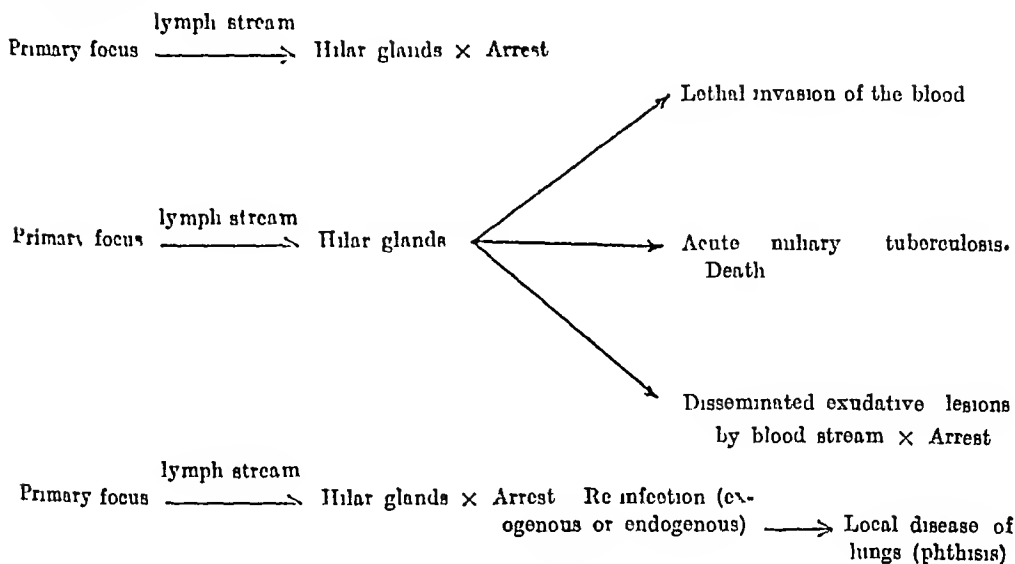
The same author classifies the childhood types of intra-thoracic tuberculosis into (*a*) a benign group, viz, epituberculosis with tubercular adenitis, ' chronic miliary tuberculosis ' (which may be associated with epituberculosis) and tubercular pleurisy with effusion, and (*b*) a severe group, viz, acute miliary tuberculosis and tuberculous broncho-pneumonia Parsons (*loc cit*) also emphasizes the fact that pulmonary tuberculosis in childhood may be either benign or severe in character

It is well known that in tropical races tuberculosis is often of the childhood type and this is sometimes attributed to absence of infection in early life and consequent non-development of immunity The severity of the disease in wild as compared with domestic animals is said to be due to the same cause That this is not, however, the sole explanation, at least as far as human races are concerned, is apparent from some recently published data Thus, although tuberculosis among adult South African negroes is apt to be severe and generalized, infection in childhood is quite common in native villages Further, in the adult American negro the childhood type of disease is prevalent and the disease is on the whole more severe than it is in the white American, yet tuberculin tests indicate the same degree of infection in early life among coloured as among white races No satisfactory explanation of these phenomena has been offered It has been suggested that acquired immunity is more frequently inherited among whites but there is little evidence to support this The hypothesis that the difference between the races is genotypic is one that is difficult to prove but is the one adopted by Pinner and Kasper (1932) who have made an extensive study of tuberculosis in the American negro

In the study and diagnosis of the childhood types of intra-thoracic tuberculosis X-rays are especially necessary Physical signs may be of little use and symptoms

are not characteristic   Without skiagraphy and examination of the sputum, when available, many cases may go undiagnosed altogether, and in patients with obvious tuberculous lesions of the lungs X-rays usually show much more extensive involvement than can be detected by ordinary methods of examination

A study of the adult cases of pulmonary tuberculosis admitted to the Mayo Hospital, Lahore, reveals the fact that the majority shows a greater degree of allergy than do corresponding cases in the West   Thus parenchymatous exudative lesions of a temporary nature may be associated with structural changes and it is not until the former clear up that the real extent and degree of the latter become evident   Plate XXXII, figs 1 (*a*) and 1 (*b*), illustrates the almost complete disappearance in 2½ months of a sharply demarcated area of consolidation (epituberculosis) in the upper lobe of the right lung revealing a chronic underlying lesion   The patient was a female aged about 30   The temperature ranged from 99°F to 101°F and the sputum contained tubercle bacilli in moderate numbers   The wedge-shaped opacity seen in Fig 1 (*b*) between the upper and middle lobes is probably due to the persistence of a small amount of exudate In some cases a more or less triangular area of disease, which seems to have extended outwards from a mass of enlarged root glands, gradually contracts from without inwards leaving a residue of ' hilar tuberculosis '   Occasionally, however, this area persists or extends further and caseation with cavitation and perhaps general dissemination may occur   Plate XXXIII, fig 2, is from a patient of this type   There was extensive breaking down of the middle of the left lung with a more or less exudative lesion at the apex of the right   The temperature ranged from 102°F to 105°F and the progress of the disease was rapid   Symptoms of abdominal tuberculosis eventually developed   An exudate into the pleural cavity analogous to the pulmonary exudate in epituberculosis would seem to have occurred in the patient from whom Figs 3 (*a*) (Plate XXXIII) and 3 (*b*) (Plate XXXIV) were taken   In this case there were marked root shadows with extensions into the lung tissue especially on the left side   When admitted to hospital the patient was having continuous fever ranging from 101°F to 103°F   The sputum contained tubercle bacilli   After a fortnight the temperature fell to 99°F to 100°F and about this time fluid was detected in the left pleural cavity   The temperature, however, instead of rising, fell with the development of the effusion and reached normal in a week

A type of pulmonary tuberculosis described as ' chronic miliary ' is sometimes seen in childhood in Western countries   In this condition there is no evidence of consolidation, the physical signs being diffuse and catarrhal   At the onset of the disease they may be absent or very slight   As a rule there is not much constitutional disturbance and the disease clears up completely in some cases   In others it progresses slowly to fibrosis, occasionally with a certain amount of bronchiectasis Skiagraphy shows rather fine, woolly stippling of the lungs, often with enlarged root shadows   Epituberculosis or pleural effusion may be present   Plate XXXIV, fig 4, is from a case that appears to have been of this type   The patient, a man aged 27, was admitted to hospital with a history of two months' illness   There had been gradual loss of weight and strength but the symptoms were never severe   The sputum was copious and contained tubercle bacilli and the temperature ranged from subnormal to 99 2°F   Diffuse catarrhal signs were present on both sides but there was no evidence of consolidation   The X-ray picture showed marked

PLATE XXXII
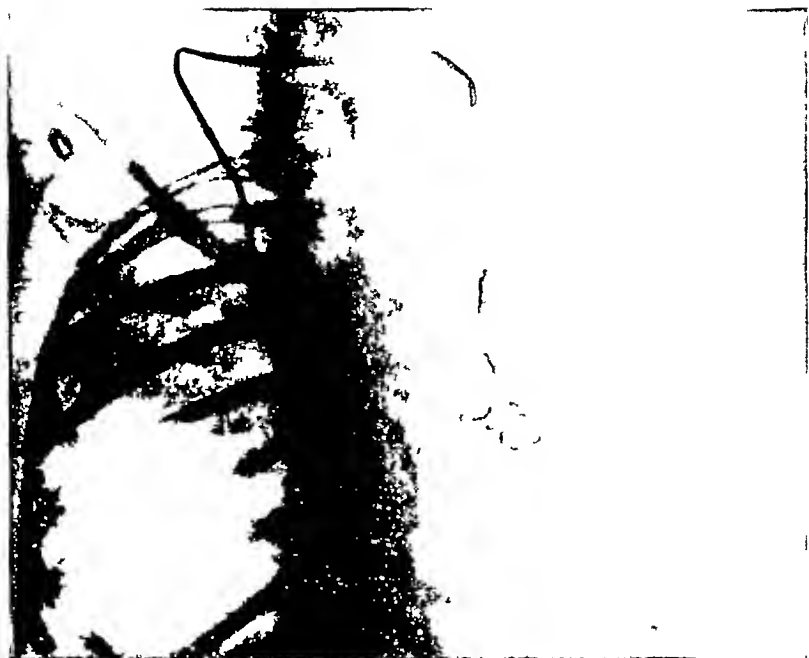
Fig 1 (a) —Female, aged 30 Diffuse infiltration of the right lung with a sharply demarcated 'epituberculous' lesion in the right upper lobe Increased root shadows

Fig 1 (b) —Same patient, 2½ months later The epituberculous lesion has almost completely disappeared A triangular shadow is present in the region of the septum separating the upper and middle lobes

PLATE XXXIII

Fig 2 —Female, aged 17 Rapidly progressing lesion in the middle of the left lung with a less dense area of disease in the upper part of the right lung The latter seems to be partly the result of parenchymatous exudation

Fig 3 (a) —Male, aged 28 Marked root shadows extending into both lungs, especially the left

PLATE XXXIV

Fig 3 (h).—Same patient, three weeks later   Large symptomless effusion in left pleural cavity.

Fig 4.—Male, aged 27   Appearances suggesting: chronic miliary tuberculosis; Marked enlargement of the root glands and thickening of the left pleura

PLATE XXXV

Fig 5 —Male, aged 21 Diffuse lesions suggestive of broncho-
pneumonia   Root glands very much enlarged

Fig 6 —Male, aged 16   Progressive bilateral disease more intense on
the left s de

PLATE XXXVI

Fig 7 —Male, aged 22 Bilateral disease with marked concentration on left side Much thickening of the left pleura

Fig 8 —Male, aged 30 Disease of both lungs of ten years' duration Evidence of extensive fibrosis Cavity in middle of right lung Enlarged hilar shadows

enlargement of the root glands, moderately fine woolly stippling of both lungs and thickening of the pleura over the middle of the left lung. With rest in bed the patient's temperature came to normal and other signs and symptoms gradually disappeared. The sputum became small in amount and free from tubercle bacilli.

In some patients with diffuse disease the mottling is much coarser in type than in the chronic miliary cases and is suggestive of a broncho-pneumonic condition. In these the physical signs are well marked. Constitutional symptoms are usually severe but arrest of the disease with extensive fibrosis sometimes occurs. Plate XXXV, fig 5, is from a case of this nature who was admitted in a very weak state with a temperature ranging from normal to 102°F. Expectoration was profuse with numerous tubercle bacilli and there were well-marked broncho-pneumonic signs. However, with rest, general symptomatic treatment and eventually sanocrysin the temperature settled to normal in the course of two months and the patient left hospital much improved with the sputum free from tubercle bacilli.

Although cases exhibiting characteristics of the childhood types of pulmonary tuberculosis in as marked a degree as those described above are not very common, they are seen more frequently than those in whom the disease is definitely ' adult ' in type. The majority lie between these extremes. In them the disease is more widespread than it is in the average case of adult tuberculosis in the West and is usually bilateral (Plate XXXV, fig 6), the greater part of both lungs being often involved. Along with this there is sometimes a concentration of the disease and an attempt at localization in a part of one lung (Plate XXXVI, fig 7), evidence of a transition from the childhood to the adult variety. The reaction to infection is, at least in the beginning, more intense than in the corresponding Western patient. This intensity may be maintained with the result that the illness runs a rapid course with high continuous or remittent fever and extensive involvement and destruction of the lung tissue, or the allergic factor may die out gradually after a while and immunity assert itself. In the latter case there is widespread formation of fibrous tissue (Plate XXXVI, fig 8), the X-ray shadows become less dense and the disease is either completely overcome or it enters into a slowly progressive stage with low remittent fever, resulting eventually in shrinkage of the lungs and some degree of bronchiectasis.

As an injection of tuberculin may produce an evanescent ' epituberculous ' condition in individuals who are very sensitive to tuberculo-protein it is possible that many transient lesions of the exudative type are produced by antigens liberated from adjacent foci of infection and contain no tubercle bacilli themselves. When, however, large amounts of antigen in the form of the actual micro-organisms are constantly present in very allergic tissues a more prolonged and intense inflammatory reaction occurs, often resulting in tissue destruction. This is what happens in the early stage of the disease in many of our patients.

It has been noticed that the proportion of severe cases is greater among female hospital patients than among male. While this may be partly due to the weakening effects of repeated pregnancies and lactation it is probably attributable, to some extent, to the fact that women, owing to their secluded methods of life, are often exposed to more continuous and intense infection than men,

As is to be expected in allergic individuals, many of whom have a relatively low degree of immunity, non-pulmonary tuberculosis is commonly associated with pulmonary disease   Infection of the lymph glands both superficial and deep is, of course, frequent and symptoms of intestinal disease are present in many patients Less commonly the larynx, bones, joints, Fallopian tubes and other organs and tissues are involved   While the intestinal disease may be the result of swallowing bacilli-laden sputum or contaminated food, it is probable that most of the complicating extra-thoracic lesions are due to infection from the lungs through the blood stream   This may occur while the invasion of the lungs is still small in extent Further, X-rays often reveal the scars of healed or quiescent pulmonary disease in the presence of active tuberculosis elsewhere   The occasional autopsy obtained shows that most patients who die with extensive and progressive lesions in the lungs have active tuberculosis in other organs   Some indication of the frequency with which the lungs are involved is given by the following figures taken from the out-patient records of the Mayo Hospital for the years 1929 to 1934 —

Yearly average number of tuberculous patients with pulmonary disease, 790 5

Yearly average number of tuberculous patients without detectable pulmonary disease, 952 8   (Figures for in-patients are not quoted owing to the limited accommodation for pulmonary cases)

It is worthy of special notice that tuberculous meningitis is very rare among these patients   As examination of the cerebro-spinal fluid for tubercle bacilli was not done it is impossible to say whether infection ever reached the subarachnoid space without causing meningeal inflammation   It has recently been contended (Rich and McCordock, 1933) that infection of the cerebro-spinal fluid in tuberculous bacillaemia is generally not sufficiently intense to cause meningitis which is said to result from local tuberculous disease of the brain   Tubercle bacilli have been found (McGregor, 1934) in the fluid of tuberculous children with little or no signs of meningeal inflammation and it is probable, in view of the widespread nature of the disease in so many patients, that bacteriological examination would reveal a similar state of affairs in some of our cases

It is not possible from the data available to give even an approximate idea of the prevalence of intra-thoracic tuberculosis among children in the Punjab as only a small number of sick children are brought to hospital for medical conditions and very few of these have tuberculous pulmonary disease   Children and young adults, however, constitute a large proportion of admission for non-thoracic tuberculosis and there is no doubt that facilities for infection are abundant   The occurrence, in spite of this, of the childhood types of the disease in adults supports the contention that the difference between races in the reaction to tuberculous infection in adult life is not entirely dependent on the degree of infection in childhood

In the treatment of these patients considerable use was made of gold preparations, especially Sanocrysin   The dangers of this method of treatment and the importance of small and carefully graduated dosage are well known   It is necessary to emphasize that gold compounds should not be used when there is any considerable degree of allergy but only when the immunity process is gaining the upper hand They exert no specific effect on the tubercle bacillus, their action being to stimulate the natural reparative processes

SUMMARY

A description is given of cases illustrating the occurrence of childhood types of pulmonary tuberculosis among adult indigenous inhabitants of the Punjab It is pointed out that, while typical cases of this kind are not common, most patients show a more intense allergic reaction to infection than do adults in Western countries

Nevertheless, some indication of immunity is nearly always present and in many patients complete arrest of the disease occurs The cause of the intensity of the reaction is discussed and it is concluded that it cannot be entirely attributed to freedom from infection in childhood

In spite of the frequency of widespread infection tuberculous meningitis is rare, at least among hospital patients

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HÆMATOLOGICAL STUDIES IN INDIANS

Part I

HÆMOGLOBIN ESTIMATION METHODS

BY

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(*An Inquiry under the Endowment Fund of the School*)

INTRODUCTION

ONE of the results of the recent hæmatological *renaissance* has been the focusing of attention on the extremely unsatisfactory nature of the usual methods both of estimating the hæmoglobin content of a patient's blood and of expressing the result of this estimation The use of the term ' hæmoglobin percentage ', without any reference to what is the standard of comparison is a curious survival in a scientific age British writers whilst being the worst offenders have a better excuse than have those in America and other parts of the world, because 13 8 grammes of hæmoglobin per 100 c c of blood has long been accepted as the ' normal ' in Great Britain , in other countries figures varying up to 17 3 grammes are taken as ' normal '

The only reasonable interpretation of the statement that a person's hæmoglobin is 100 per cent is that a given quantity of his blood contains an amount of hæmoglobin equal to the mean hæmoglobin content of the blood of an infinite number of healthy adult males of the same race, living under the same meteorological conditions One cannot, therefore, conceive what was the process of reasoning of the writer of the following paragraph, which is quoted from a recent textbook on medicine —' The normal blood count of an adult is as follows — Hæmoglobin 80 to 90 per cent '

The only, and the obvious, alternative is to give the hæmoglobin content in the terms of grammes per 100 c c of blood , this practice is now being adopted by most

(809)

American writers, but British writers are hesitating because, they say, clinicians have in the past been taught to think in terms of percentages—but, we would add, percentages of an arbitrary and varying standard '. This is a defeatist attitude, and quite inexcusable

It is difficult to explain the comparatively low ' normal ' hæmoglobin content of blood in Great Britian , there is, however, no doubt that the difference between the British and American ' normals' is real , this difference has led to much confusion in the calibration of instruments, but the confusion would never have arisen except for the present ridiculous method of recording results, and it could now be almost entirely obviated were results expressed in terms of grammes per 100 c c of blood, and instruments calibrated accordingly

METHODS OF HÆMOGLOBIN ESTIMATION.

The remedy for the unsatisfactory method of expressing results is comparatively simple, but the difficulties arising out of variations in the method of estimating hæmoglobin are not so easily overcome   In the first place there is no uniformity of opinion as to which particular property of the hæmoglobin should be measured The properties that have been measured include the colour, the oxygen-combining power, the iron content, and the refractometric index   It is assumed that hæmoglobin is a fixed molecule and that, therefore, its properties will be constant , it should, therefore, not be a matter of great importance which property is measured, provided the formula necessary to convert the measurement we make into terms of grammes of hæmoglobin has been worked out   Clinical methods of estimating hæmoglobin are all colour methods, and, although the instruments supplied by good manufacturers should be satisfactory for clinical work, if any scientific investigation is to be undertaken it is imperative that the instruments used should be checked by some method that measures one of the other properties of hæmoglobin

Colorimetric methods —Their number is legion, but those with which we have had personal experience can be classified as follows —

  I   Direct comparison of colour of undiluted blood against a graduated colour standard—

  (a) On special blotting-paper (Tallqvist)

  (b) Between glass-plates (Dare)

  II   Comparison of blood diluted to a fixed percentage against a graduated colour standard

  (a) Diluted with sodium carbonate solution and compared with a coloured glass wedge (Fleischl-Miescher)

  (b) Diluted with deci-normal hydrochloric acid and compared with a coloured glass wedge (Hellige-Neoplan)

  III   Comparison of blood diluted to a varying degree with a fixed colour standard

  (a) Diluted with water and compared to a permanent picro-carmine standard (Gowers)

(*b*) Brought into contact with carbon monoxide, diluted with water, and compared with a permanent colour standard (Haldane)

(*c*) Diluted with deci-normal hydrochloric acid and compared with a permanent standard of acid hæmatin (Sahli),' or a coloured glass-block (Hellige)

IV   Comparison of blood diluted with deci-normal hydrochloric acid to a fixed percentage against a fixed colour standard in a colorimeter of the Duboscq type

(*a*) Water placed in one chamber of the colorimeter and a standard coloured disc interposed (Newcomer)

(*b*) Compared with an acid hæmatin solution of known strength

DISCUSSION

It will be possible to discuss the advantages and disadvantages of some of these methods without going into elaborate details

I*a*   The Tallqvist is the easiest of all methods, but is the least accurate   The sources of error are obvious and numerous , they included variations—in blotting-paper and its powers of absorption, in the colour standards, in the size of blood drop taken, and in the observer's sense of colour

II*a*   The Fleischl-Miescher in the hands of some workers appears to give comparatively uniform and satisfactory results, but we [the senior writer (L E N) in particular] find great difficulty in comparing the intensity of two colours that do not match one another   Using this instrument under the very best circumstances we found that, though each of us could make five readings that showed little deviation from their mean, the means of the two sets of readings differed considerably   Further, we found that in a very short time the colours underwent a very marked change so that readings had to be done very rapidly or the time factor taken into consideration   The Fleischl-Miescher readings that are referred to below were made by a colleague who constantly uses this instrument, and who gets more consistent results than we were ever able to get   In each case he took 10 readings—5 in each chamber and his figure was the mean of these 10 readings

This inability to match depths of colours that are qualitatively different has been our main stumbling block in all the methods that we have tried, except the acid hæmatin methods

The method IV*b* in the above classification is one that we hoped would give us good results, but we failed to get a standard that would match with our blood solutions

IV*a*   The apparatus of the Newcomer type that we used is a good instrument made by a well-known American firm of opticians, but we found that change in the intensity of the daylight made great differences to our readings , with artificial light we did not get any more satisfactory results   Further, the scale is only marked in grammes, and as the distances between the marks decrease rapidly at the top

of the scale, it is difficult to guess fractions of a gramme accurately, especially in the higher readings   A number of readings were made with this instrument in conjunction with the other methods but, as the results appeared irregular and the readings made by different observers showed a wide disparity, they are not reported

IIIc   The great advantage in the Hellige instrument is that it is possible to match the acidulated blood solution with the fixed standard exactly, and, as the latter is made of coloured glass, it is unlikely to undergo any colour change   The sources of error, excluding those due to carelessness in technique, using dirty pipettes, etc , will arise through variations in the diluting pipettes and in the calibres of the graduated tubes   The method suffers the great disadvantage that with each dilution only one reading can be made—a second observer can check the first reading, but not make an independent one—and that the time interval before reading has to be taken into consideration   (The error caused by the time factor does not exceed 1 per cent and is in no way comparable to that we noted with the Fleischl-Miescher instrument)

IIb   In the Hellige-Neoplan instrument the sources of error are again the diluting pipette and the chamber in which the acidulated blood is placed, but, as the latter is a rectangular chamber made with flat pieces of glass cemented together, precision in its manufacture is easier than it is in the case of the graduated tubes of the other type of Hellige instrument   Further, any number of readings can be made with the same specimen and thus one source of error can be practically eliminated   This is very necessary in the instrument because we have found that matching is not so exact, particularly at either end of the wedge   Besides this, theoretically it would be possible for this type of apparatus to give correct readings at one end of the wedge and incorrect at the other, whereas with the fixed standard of the other type if it is correct for one percentage it will be correct for all

The utility of the different methods is an important point and only the colorimetric methods can be said to be clinical or field methods   Of the methods with which we are concerned in this paper, the Hellige, the Hellige-Neoplan and the Newcomer are methods that require no special conditions , the apparatus for the last named is larger and more delicate than that of the other two, and is therefore less suitable for field work   The Fleischl-Miescher requires a dark room or at any rate a dark box, and an oil lamp, and is, therefore, even less suitable for field work

THE HALDANE-VAN SLYKE OXYGEN METHOD

The oxygen-combining power of the blood is the property usually accepted as the best for calibrating clinical hæmoglobin-measuring appliances, on the grounds that on this property depends one of the main functions of hæmoglobin, that the oxygen-combining power of the other elements of the blood are negligible, and finally that it is possible to make the estimation with a considerable degree of precision   Unfortunately, there was no van Slyke apparatus available in the institution at the time we commenced these investigations and we felt that it might lead to a considerable waste of time, possibly without obtaining satisfactory results, to

undertake this estimation under sub-optimal conditions, we did not therefore use his method in this series*

The iron-content method of Wong—This method depends on the observation that, though its graphic formula has not yet been worked out, hæmoglobin is known to have a constant molecule containing a constant percentage of iron   It has also been shown that the extra-hæmoglobin iron in blood is a small constant factor constituting about 2 per cent of the total blood iron   There have been many recent references to this method by American workers who all appear to have obtained satisfactory results, and, as the method appeared to present no special difficulties, we adopted it for checking the colorimetric methods

However, we were not satisfied with our results and after cross-checking them by the refractometric method we found that they were consistently too high   This was surprising in view of the fact that, though our reagents were 'guaranteed iron-free', we had tested them all carefully before commencing the work and had done a number of 'blank' estimations   Eventually we discovered that the filter-paper in most of our experiments contained a large amount of iron , all our results were, therefore, vitiated and we have had to exclude them from this paper

The refractometric method—Again, this method is dependent on the constancy of the hæmoglobin molecule , by taking the refractometric index of a solution of hæmoglobin it is possible to calculate the concentration of the hæmoglobin present   As a good quality dipping refractometer was available we adopted this method to check the different colorimetric methods in a series (B) of blood samples from 31 different persons

THE CLINICAL MATERIAL

Certain hæmatological investigations were being carried out on normal Indians living in Calcutta, normal tea-garden coolies in Assam, and on persons suffering from anæmia in both localities   The hæmoglobin estimation discussed here constituted part of those investigations   Series A were all apparently healthy adult Indians, doctors, clerks and laboratory attendants, and servants working in the Calcutta School of Tropical Medicine

Series B were other persons from the same source, but in addition this included some non-anæmic patients from the hospital attached to this institution and some anæmic patients under investigation

Series C were tea-garden coolies in Assam

TECHNIQUE OF THE DIFFERENT METHODS AND THEIR EXPERIMENTAL ERROR

As we have obtained results that differ from those of certain other workers it is essential that we should detail our technique, particularly with reference to the special precautions we took

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* More recently, Dr H L C Wilson of the All India Institute of Hygiene and Public Health, Calcutta, has carried out Hellige estimations on the same sample   The results we obtained suggest that our figure, 13 67 grammes = 100 per cent Hellige, is approximately correct

Hellige—Deci-normal hydrochloric acid is placed in the graduated tube up to the 10 per cent mark , blood is taken up in the special pipette to the 20 c c mark , the point of the pipette is wiped clean so that no blood remains on the outside, the contents are then transferred to the graduated tube containing the acid, and the pipette is washed out with this acid *The tube is allowed to stand for* 15 *to* 20 *minutes* , it is then placed in the Hellige comparator and water is added drop by drop until the acid hæmatin solution exactly matches the standard , the height of the lowest point of the meniscus, with the tube held level with the eyes, is then noted and the reading taken from the percentage figures marked on the tube   The contents of the tube must be mixed with the glass-rod provided for the purpose after each drop of water is added, particular care being taken that as little fluid as possible is allowed to adhere to the rod after each mixing and that the rod is held in the hand and not placed on blotting-paper, so that such fluid as does adhere to it will not be soaked up between each stirring

Changes in the colour of the acid hæmatin solution are said to take place up to the end of one hour, but we found that practical disadvantages in delaying the readings for a longer period were so great that we adopted the standard time interval of 15 to 20 minutes , the change that occurs after 15 minutes was certainly less than 1 per cent

The blood was taken (*a*) by pricking the finger, following momentary congestion produced by rapidly wrapping a piece of tape round the middle phalanx, or (*b*) from the vein at the bend of the elbow, temporary dilatation of this vein being produced by an assistant encircling the limb with his hands , in the former case the blood was taken directly into the measuring pipette, but in the latter a drop of blood from the syringe was placed in a watch-glass and from this samples immediately taken into the measuring pipettes for the Hellige and Hellige-Neoplan estimations, and the rest put into an oxalate tube (*c*) which was immediately rolled between the hands to ensure complete permeation of the whole sample by the oxalate   All the other hæmoglobin estimations were made from this sample which was always very well shaken to ensure complete mixing before the smaller samples from it were taken

The accuracy of the individual readings —The possible sources of error, independent of the correctness of the colour standard and the method of taking the blood, are   incorrectness in the calibration of the pipettes, incorrectness in the calibration of the tubes in which the diluted blood is placed, errors in filling the pipettes, and errors in matching the standard   (We are leaving out of consideration gross errors of technique due to improper cleaning of glass-ware, etc)   The former two will be constant errors for each pipette and tube , they are susceptible to measurement   The latter two will vary with each estimation that is made and will be dependent to some extent on the accuracy of the worker and also on his sense of colour* , as only one reading can be made with each specimen, it will not be possible to separate these last two sources of error   Multiple estimations were done on the same samples of blood to show the extent of the importance of these several errors, and the results are analysed below —

Six graduated tubes and three pipettes were marked *a, b, c, d, e, f,* and *x, y* and *z*, respectively   Six estimations were done on each of 23 specimens of blood of

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* All the Hellige readings recorded here were made by one observer

different persons (from series A) the following combinations of tubes and pipettes being used , x-a, x-b, y-c, y-d, z-e, z-f The means of each group of readings are given below —

Tubes	Pipette x	Pipette y	Pipette z
a	98 70		
b	95 83		
c		97 48	
d		97 35	
e			97 26
f			97 00
Means	97 265	97 415	97 13

The difference between the means of the observations on each of these sets of 23 pairs of samples taken with these three different pipettes is so small that it seems unlikely that the difference between any two will be significant   Fisher's ' t ' test can be applied to the means of the 23 pairs of readings with the pipettes y and z The difference in the means is 0 285 , t = 0 851 , P = 0 4 , the difference is, therefore, not significant

On the other hand, the means of the 23 readings with the same pipette x, but with different tubes, a and b, are appreciably different from one another, the difference being 3 13   Applying Fisher's ' t ' method, t = 2 68 and P = between 0 02 and 0 01, so that the difference is quite definitely significant   If either of the groups a-x or b-x are compared with any of the other four groups, the means will also be found to be significantly different , we applied the ' t ' test between groups b-x and c-y, and found that, though the difference in the means was less, i e , 1 65, t was actually larger, and P was less than 0 01

The means of the readings in the case of the other four tubes, c, d, e and f, show very slight differences from each other   Testing the significance between, e and f, we find that $\bar{x}$, the mean of the differences, = 0 261, t = 0 685 and P = 0 5, and between c and f, $\bar{x}$ = 0 480, t = 1 15 and P = about 0 25 , that is, there is no significance in either of these differences

There is thus every reason to believe that the three pipettes x, y, and z, and the tubes c, d, e and f are accurate and are not responsible for introducing any error into the readings , it follows, therefore, that any errors detected in readings made with

these tubes and pipettes must be due to the other sources of error, that is, to the matching of the colours or to the filling of the pipettes, great care was exercised in filling the pipettes accurately and it is probably not an important source of error, but the error due to matching the colour might be serious

The extent of this combined error can only be ascertained by taking a large number of pairs of observations on different specimens of blood, the differences observed will form a normal dispersion, the extent of which can be calculated   It will be best to express the deviation from the mean of these observations, as a standard deviation, this can be obtained from the formula $\sqrt{\dfrac{S(x_1 - x_2)^2}{2N}}$ which is applied by Tippett (1931) to the particular case of pairs of variates The sum of the squares of the differences between 138 pairs of observations in this case was 573, $\sqrt{\dfrac{573}{276}} = \sqrt{2\ 07} = \pm 1\ 44$ per cent, which figure may be taken as an expression of the experimental error of the method *

Venous, oxalated and capillary blood —As we have said above, the hæmoglobin estimations were done by the Hellige method on three samples of blood from each patient, on (a) capillary blood, (b) venous blood, and (c) oxalated venous blood It is important to find out if there are any differences in the readings of the blood taken from these different sources   they were, therefore, recorded and are examined below —

Comparison of direct venous and oxalated venous blood —In 23 cases the maximum disparity between these two readings in the same persons was 8 per cent, but this was only recorded once, a 5 per cent difference was recorded twice and the remainder were 4 per cent or below   The standard deviation of these disparities is $\pm 3\ 19$ The mean of the differences was $+ 0\ 39$ in favour of the oxalated samples   By applying Fisher's 't' method for the significance of the difference of the means, it was found that 't' in this case was $0\ 585$, $n = 22$, and $P =$ between $0\ 5$ and $0\ 6$, and therefore, the difference is not significant

Comparison of the capillary and venous (oxalated) blood —The double estimation was done in 18 cases (excluding the European and anæmic cases), the mean difference in favour of the capillary blood was 7 11 per cent   It is quite obviously unnecessary to apply any statistical tests for significance, as in every pair of estimations but one (in which the two estimations were equal) the capillary blood showed a higher value   If the difference is reduced to 0 11 by adding 7 to each of the venous blood readings, and the standard deviation calculated by the formula $\sqrt{\dfrac{S(x_1 - x_2)}{2N}}$, it is found to be $\pm 2\ 77$ which is within the range of the experimental error of the method when unselected tubes are used

Our observations with the Hellige apparatus can be summarized thus —

Taking the ordinary pipettes and selected graduated tubes we can calculate that two-thirds of the observations made will fall within 1 44 per cent of the mean

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* We do not know of any recognized method of expressing the inaccuracy of any method of hæmoglobin estimation, but it seems to us that the standard deviation of a number of readings on the same blood is one that expresses this fairly satisfactorily   It has seldom been possible actually to make a number of observations on the same blood, but this value has been arrived at by other means, usually by taking the differences between pairs of observations.

of all the observations, and that if an observation on a specimen of blood differs from that on another specimen by more than $2 \times (1\ 14)^2$, $= 4\ 07$ per cent, the hæmoglobin value of these bloods may be said to be significantly different (on a 20 to 1 basis) To these figures have to be added the bias of the tubes and pipettes In our experiment four out of six tubes, and all the pipettes appear to be accurate, and the bias in the other two tubes appeared to amount to about 1 5 per cent each So that these two figures will become $(1\ 44 + 1\ 5) = 2\ 94$ and $(4\ 07 + 1\ 5) = 5\ 57$, respectively

The addition of a small amount of oxalate to the blood makes no significant difference to the hæmoglobin estimation

There is a very marked difference between hæmoglobin content of the capillary and the venous blood in the normal Indian In a series of 18 normal persons the capillary blood was 7 11 per cent higher

THE HELLIGE-NEOPLAN APPARATUS

Method —Special diluting pipettes are supplied with this apparatus but the ordinary red-blood-counting pipettes can be used Blood is drawn up to the ' 1 ' mark and then deci-normal hydrochloric acid up to the ' 101 ' mark The reading is taken after the same interval as in the last method—15 to 20 minutes was the time we allowed The portion in the stem of the diluting pipette is discarded and the rest of the acid hæmatin solution is placed in a glass chamber which is then placed in position in the apparatus and compared with a coloured glass wedge The point at which it matches the coloured wedge is read off on a scale at the side At least five independent readings should be made, and the mean of these five readings taken as the final reading

Sources of error —These, as we have said, are the diluting pipettes, the chambers in which the acid hæmatin solution is placed, and the matching of the colours

We used only two chambers in our experiments, and we found no significant difference between the means of two sets of figures done on the same series of cases

The error due to matching is very easily found out, as it is possible to do any number of readings on one specimen It would not have been advisable to take a hundred readings from one specimen as the ' fatigue ' factor would come into play, so we took a number of pairs of readings on various specimens For 120 pairs whose mean was 88 64 per cent the standard deviation was $\sqrt{2\ 25}$ or $\pm 1\ 5$, and the standard error of the mean of the five readings $\sqrt{1\ 5}$ or $\pm 0\ 67$

Four pipettes were numbered, as with the previous method, and counts done with each of the pipettes on a number of different bloods , the means of 30 readings with the four different pipettes were, respectively, 107 3, 105 5, 108 7 and 109 2 per cent Applying the ' t ' significance test, we find that the mean of the readings with the second pipette is significantly different from that of the third and fourth pipettes, but that the others were not significantly different from one another,

We took 36 pairs of estimations, each of which was the mean of five readings, and by applying the formula $\sqrt{\dfrac{S(x_1 - x_2)^2}{2N}}$ calculated that the experimental error of this method amounts to $\pm 3\,00$ per cent

The error due to matching the colour with the standard only amounts to $\pm 0\,67$ so that most of this error will be due to irregularities in the calibration of the pipettes and could probably be reduced if only accurate pipettes were used

The difference between the venous and capillary blood was noted also with this instrument , the difference in the means of 24 readings being 8 54 per cent in favour of capillary blood

THE REFRACTOMETRIC METHOD

We adopted the method described by Stoddard and Adair (1923) , the technique is as follows —

1   Measure two c c samples of blood into two medium sized test-tubes
2   To one sample add 5 c c of 0 8 per cent NaCl
3   To the other sample add 5 c c of 0 8 per cent NaCl containing about 70 mg of saponin per 100 c c
4   Centrifuge (2) and (3) and read the clear solutions in the refractometer   Read also the two salt solutions

Calculations —Subtract the refractive indices of the two salt solutions
This gives the added refraction due to the saponin

Multiply by $\dfrac{5}{5\,6}$ to get the dilution of the saponin refraction by the added 0 6 c c (approximately) of plasma   Subtract this saponin correction from (3) and then subtract (2) from the result   This gives the increase in refraction due to the hæmoglobin   Divide by the hæmoglobin oactor, or 194 2, and multiply by 6 (the dilution) to get the concentration of hæmoglobin in the friginal blood.

Of course, the refractive index of the salt and the saponin solutions need not be determined for every hæmoglobin determination   The saponin tends to precipitate somewhat with age, so that it is advisable to check it up from time to time

It was found quite impossible to carry out these readings at ordinary room temperature even using a water-bath   A dipping refractometer, made by Zeiss, was used   The refractometric index was worked out, in the conversion tables supplied with the instrument, for 17 5°C , the temperature of the ' cold room ' is about 23°C , and it was necessary to wait at least 6 minutes between each reading for the instrument to acquire the temperature of the water-bath   We used a stop-watch and wrote down the readings at minute intervals until they ceased to undergo any change   Each writer took an independent reading with each solution, and, if they were different, the mean of these two readings was taken   With the serum and salt solutions the readings were clear-cut and there was seldom any disparity between those of the two observers , with the hæmoglobin solution there was often some disparity but this never amounted to more than 0 1 of the scale (this corresponds roughly to 0 11 gramme of hæmoglobin) and in most cases it was 0 05 or less   The whole procedure was performed in duplicate , the largest disparity between any pair of readings was 0 41 gramme, and the standard deviation of the readings was

$$\sqrt{\dfrac{S(x_1 - x_2)^2}{2N}} = \pm 0\,145 \text{ gramme, or just over one per cent}$$

We estimated the hæmoglobin by the refractometric method in 31 persons (series B) carrying out estimations by two or more of the colorimetric methods on

the same blood The mean of the two estimations was taken as the final figure The 100 per cent of the Hellige method corresponds to 13 67 grammes by this method

*The refractometric method compared with the Hellige, Hellige-Neoplan and the Fleischl-Miescher —*In series B (*see* Table) the results obtained with the refractometric method are compared with those obtained by the three colorimetric methods It was first necessary to work out the gramme equivalent for 100 per cent with each instrument This was worked out in each individual case by dividing the refractometric finding by the percentage finding and multiplying by 100 The mean of the whole series was then taken This step is not shown in the Table , the results obtained were as follows —

$$
\begin{array}{llll}
\text{Hellige} & 100 \text{ per cent reading} & = 13\,67 & \text{grammes} \\
\text{Hellige-Neoplan} & \text{,,} \quad \text{,,} \quad \text{,,} & = 14\,79 & \text{,,} \\
\text{Fleischl-Miescher} & \text{,,} \quad \text{,,} \quad \text{,,} & = 13\,07 & \text{,,}
\end{array}
$$

The individual readings with the three colorimetric instruments were then converted into grammes by multiplying these three figures by the percentage findings and dividing by 100 We then had four readings each made with a different apparatus, and, as it was assumed that the refractometric method was the most accurate, the readings with each or the other apparatuses were first compared with the refractometric readings Using the formula $\sqrt{\frac{S(x_1 - x_2)}{N - 1}}$, the standard deviations of the individual differences were calculated , these are as follows —

	31 pairs	30 pairs	29 pairs
Refractometer and Hellige	± 0 569	± 0 546	± 0 552
Refractometer and Hellige Neoplan		± 0 776	
Refractometer and Fleischl Miescher			± 0 505

This suggests that the Fleischl-Miescher apparatus is the most accurate or at least gives results with the least degree of disparity from those of the refractometric method , the Hellige is not far behind it in accuracy, but the Hellige-Neoplan appears to be considerably less accurate This is a conclusion that we had already arrived at

The refractometric reading is the mean of the readings of two samples of which the experimental error of each is 0 145 gramme, and the others are readings on single samples The disparities between the refractometric method and the Hellige are only a little greater than could be accounted for by the experimental error of the separate methods

In 13 instances in this series five additional Hellige readings were taken, and if the mean of the six readings, instead of the first reading only as given in the Table, is taken and compared with the refractometric reading, it is found that the standard

deviation of the differences is now only ± 0 420 gramme , and, if the one exceptionally high figure (1 30 grammes) is excluded, the standard deviation of the remaining 12 differences is reduced still further to ± 0 224 gramme

Hellige and Hellige-Neoplan readings —Taking the readings of series B (*see* Table), there is a difference in the means of the Hellige and Hellige-Neoplan readings of 6 40 per cent in favour of the former   When the readings are reduced to grammes the differences in the means of the two series is naturally eliminated, but the standard deviation of the differences in the individual pairs of readings is ± 0 623 gramme , that is to say there does not seem to be any closer correlation between these pairs of readings made by different colorimetric methods, than one would expect from the observed disparities between each of them and the refractometric readings

TABLE

Showing hæmoglobin estimations by various methods (Series B)

| | FINGER BLOOD. | VENOUS BLOOD | | | | | | | |
| | | DIRECT | OXALATED | | | | CALCULATED IN GRAMMES | | |
	Hellige	Hellige	Hellige	Hellige Neoplan	Fleischl	Refractometer	Hellige	Hellige Neoplan	Fleischl
N 1	110		101	97	111	14 78	13 85	14 35	14 57
N 2	115	105	102	100	118	14 60	13 98	14 79	15 49
N 3	132	125	127			16 35	17 41		
N 4	105	104	100	94	112	13 49	13 71	13 90	14 71
N 5	122		113	111	122	15 79	15 50	16 42	16 02
N 6			97	90	100	13 29	13 30	13 31	13 13
N 7	106	95	98	94	100	13 32	13 44	13 90	13 13
N 8	102		90 5	84	92	12 28	12 41	12 42	12 08
N 9			102	102	107	15 01	13 98	15 09	14 05
N 10			90	82	105	13 60	12 34	12 13	13 79
N 11			130	128	131	17 33	17 82	18 93	17 20
N 12	112	104	105	97	108	14 32	14 40	14 35	14 18
N 13	118	110	114	104	117	15 44	15 63	15 38	15 36
N 14	122	118	122	114	120	16 50	16 73	16 86	15 76
N 15	106	100	100	95	108	14 31	13 71	14 05	14 18
N 16	102	100	99	92	103	13 76	13 57	13 61	13 52
N 17	104	104	102	97	112	14 47	13 98	14 35	14 71
N 18	134	124	126	121	124	16 18	17 27	17 90	16 28
N 19	142	128	127	130		17 67	17 41	19 23	
N 20	120	108	112	107	116	14 97	15 35	15 83	15 23
N 21	134	127	122 5	112	127 5	16 31	17 48	16 56	16 74
		110	118	110	121	15 83	16 18	16 27	15 89
		127	132	127	136	16 91	18 10	18 78	17 86
		81	81 5	74	80	10 76	11 17	10 94	10 50
			71	67	76	9 64	9 73	9 91	9 98

TABLE—*concld*

| | FINGER BLOOD | VENOUS BLOOD | | | | | | | |
| | | DIRECT | OXALATED | | | | CALCULATED IN GRAMMES | | |
	Hellige	Hellige	Hellige	Hellige Neoplan	Fleischl	Refracto meter	Hellige	Hellige Neoplan	Fleischl
A 3	72	68	67	60	72	9 82	9 19	8 87	9 45
A 4	33	29	27	23	31 7	3 60	3 70	3 40	4 16
A 5	35	33	34	31	31	4 70	4 66	4 58	4 07
A 6	32	27	26 5	22	31	3 63	3 63	3 25	4 07
A 7	61	61	60 5	52	58	8 25	8 30	7 76	7 62
A 8	29	26	29	23	27	3 74	3 97	3 40	3 55

In a series of 240 pairs of observations on 240 coolies on Assam tea-gardens at an earlier date (from series C), there was a difference of 2 35 per cent in the means of the Hellige and Hellige-Neoplan series, respectively, the actual figures being 71 35 per cent and 69 00 per cent , these correspond to 9 846 grammes and 9 660 grammes calculating from the reputed hæmoglobin equivalents of the two apparatuses, namely, 13 8 and 14 00 grammes per 100 c c of blood , this shows an unimportant disparity in these two sets of readings

On the other hand our later observations (series B *vide supra*) show a much greater disparity between the Hellige and the Hellige-Neoplan readings, namely 6 40 per cent, and we must conclude that the colour standard in one or other has undergone a change   The most likely change would be for one colour standard to fade, but we have compared the colour standard of the Hellige instrument that has been in use for some time with one that has not been used and can find no significant difference , further, our recent observations suggest that at present the 100 per cent with this apparatus corresponds to 13 67 grammes of hæmoglobin   Even if this difference between 13 67 and 13 80 indicated some slight fading, it would be quite insufficient to account for the differences noted above   On the other hand the figures in the Table indicate that at that time 100 per cent on the Hellige-Neoplan corresponded to 14 79 grammes of hæmoglobin, as against the reputed 14 00 grammes which was more or less confirmed in our Assam series of observations, and we believe that the colour standard in this apparatus has undergone a change , it seems possible that the material used for sticking the glass wedge has become darker, in the way that the material used between the layers of ' triplex ' glass takes on a brown colour in this climate

The Hellige and Tallqvist methods —Double estimation, by the Tallqvist and the Hellige, were made in 478 coolies on 18 different tea-estates (series C)   About three-quarters of these were taken from the normal ' healthy ' population and the remainder were anæmic patients attending the hospitals on the tea-estates.

The Tallqvist scale has 10 divisions, from 10 to 100 per cent, but when it is considered that a specimen is darker than one colour, yet lighter than the one above

CHART

Showing the individual readings in 240 cases in which the hæmoglobin was estimated by both the Tallqvist and Hellige methods

Per cent readings by Hellige normal hæmometer.
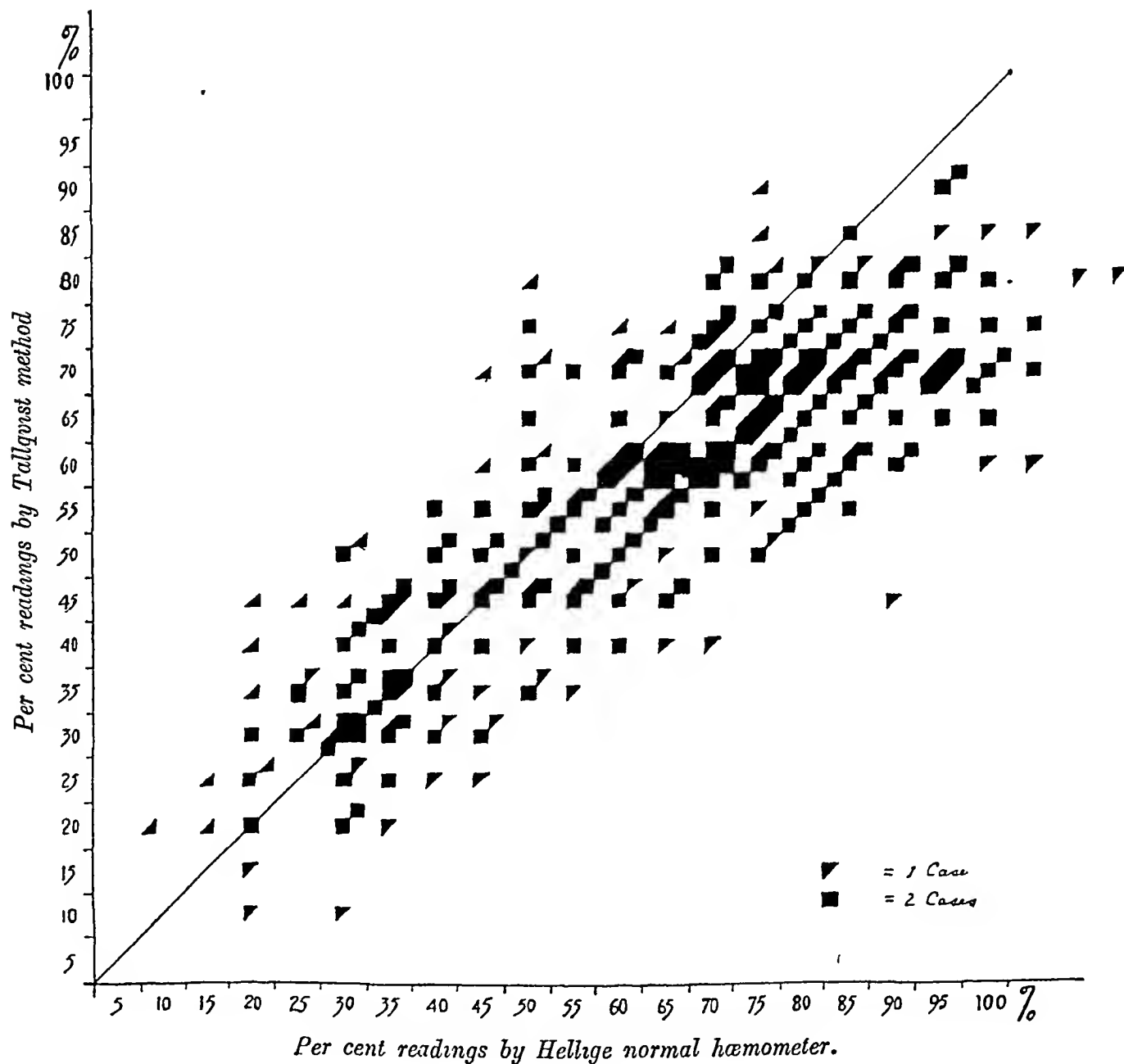
it, an intermediate reading is made , all the readings will, therefore, be multiples of five It was found that there was only a slightly greater tendency to make a reading at the even figure (10 per cent, 20 per cent, etc) rather than at the intermediate figure (5 per cent, 15 per cent), the ratio being 56 to 44

GRAPH

Showing the mean and standard deviation of the Hellige readings for cases grouped according to their Tallquist readings

Per cent readings by Hellige normal hæmometer
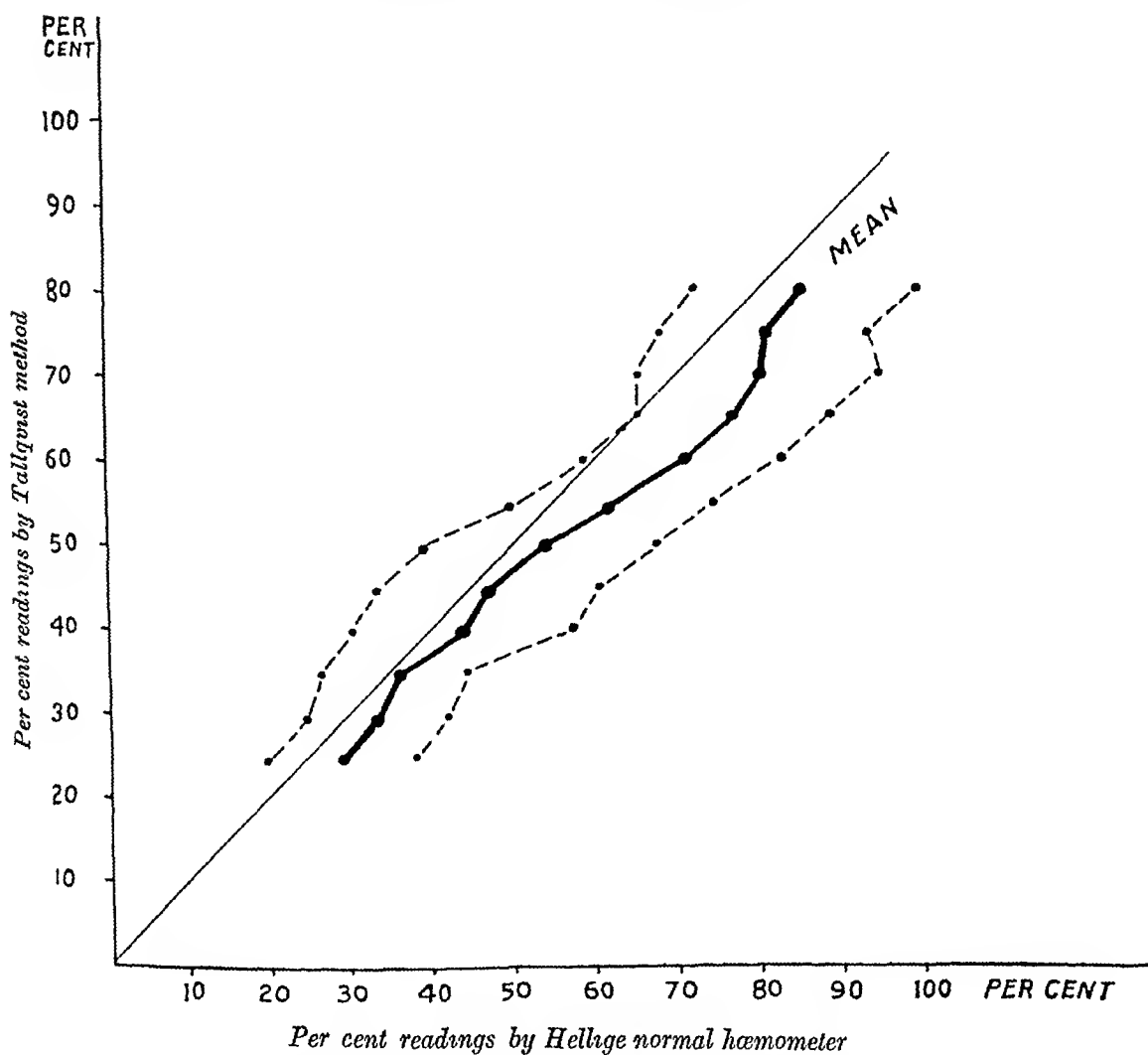
The Hellige scale is divided into one-per-cent divisions and the readings were made to the nearest per cent, but for these calculations we have grouped them to the

nearest 5 per cent , that is to say, readings from 8 to 12 per cent are classed as 10 per cent, those from 13 to 17 per cent as 15 per cent, and so on   The results are entered on the Chart   The readings were then grouped according to the Tallqvist readings and the mean and standard deviation of the Hellige readings of each group were worked out , these are plotted on the Graph   It will be seen that the mean of the Hellige readings in every group is higher than the Tallqvist , up to 50 per cent the deviation of the means is not great, but from thence up to 70 it averages nearly 10 per cent, and again it declines slightly in the higher percentages   The standard deviations in the various groups is never less than $\pm$ 8 4 and is as high as $\pm$ 14 5 in one group , this standard deviation represents the standard deviation of the differences between the readings with the two different methods after the necessary adjustment has been made for the differences in calibration of the two apparatuses   The mean of the standard deviations of 13 groups is $\pm$ 11 73, or taking into consideration the number in each group, it is $\pm$ 12 15   This latter figure may be taken as representing the combined error of the Tallqvist and the Hellige methods, but as the error with the Hellige method may be placed at a maximum of $\pm$ 3 per cent, that leaves the Tallqvist at $\sqrt{(12\ 15)^2 - 3^2} = \pm$ 11 8 per cent   This means that the differences in pairs of observations on the same samples by the Tallqvist method will have a standard deviation of over 16 per cent and that even a difference of 30 per cent in two readings cannot be considered as quite significant

It was felt that this lumping together of all the Tallqvist readings made by a number of different observers might give an exaggerated idea of the inaccuracy of the method   We, therefore, looked for instances in which one observer had made a number of Tallqvist readings at one figure and we then took the mean and standard deviation of the corresponding Hellige readings (e g , one assistant medical officer returned 8 readings as 60 per cent , the Hellige readings on these 8 persons were 57, 62, 66, 68, 72, 72, 73, and 80 per cent , the mean and standard deviation of these is 68 9 $\pm$ 7 2 per cent)   There were 13 instances in which seven or more readings were made at the same figure by one assistant medical officer , in these 13 instances the standard deviations varied up to $\pm$ 17 35 per cent and the mean was $\pm$ 10 54 per cent   If this figure is compared with the 12 15 per cent above it shows that slightly more accuracy can be expected when the readings of only one observer are taken into consideration, but the error of the method is still above $\pm$ 10 per cent

SUMMARY

A protest is made against the present method of expressing the results of hæmoglobin estimations in terms of percentages

The different methods of hæmoglobin estimation are discussed

The technique of certain methods is described and an attempt is made to gauge the accuracy of these methods ; the standard deviation of a number of estimations on the same blood was used to express the experimental error of a method

The Hellige apparatus was found to have an error of $\pm$ 1 44 per cent ($=$ 0 197 gramme of hæmoglobin) if especially selected tubes were used   Four out of six tubes were found to be satisfactory , the other two each gave rise to an additional error of 1 5 per cent ($=$ 0 205 gramme of hæmoglobin)

The Hellige-Neoplan apparatus had an experimental error of $\pm 3\ 00$ per cent

The refractometric method of estimating hæmoglobin was used for checking the three colorimetric methods, the Fleischl-Miescher, the Hellige and the Hellige-Neoplan , the figures indicate that their order of accuracy is as above   There is no closer correlation between the readings by the different colorimetric methods than one would expect from the disparities observed between each and the refractometric method

The experimental error of the Tallqvist method, taking readings made by different observers, was 11 80 per cent, and by a single observer over 10 per cent

There is a marked difference between the hæmoglobin content of venous and capillary blood of normal Indians , the latter is 1 0 to 1 4 grammes per 100 c c of blood (7 to 10 per cent) higher

The addition of a little potassium oxalate makes no difference to the hæmoglobin estimation results

CONCLUSION

That provided the colour standard, the tubes and the pipettes of the Hellige hæmoglobin apparatus are checked by some absolute method of hæmoglobin estimation, it is a method that can be adopted for clinical purposes, or for obtaining scientific data under field conditions

That the Tallqvist method is one of little value for clinical purposes, as a difference between two readings of as much as 30 per cent cannot be considered as ' significant '

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THE INCIDENCE OF HEPATIC CIRRHOSIS IN SOUTH INDIA*.

BY

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THERE are certain observations to indicate that, in tropical countries, there is a high incidence of hepatic cirrhosis Rogers (1930) mentions the incidence of cirrhosis of the liver in Bengal as 6 9 per cent in 489 autopsies in Calcutta, whereas Berlin figures indicate an incidence of only 1 per cent in 3,200 autopsies Snijders (1933) has pointed out that mortality from cirrhosis is ten times higher in Javans than in an European population of the same age and sex in Holland The death rate from cirrhosis was 43 per hundred thousand of population in Java as compared with 4 4 in Holland Tirumurti and Radhakrishna Rao (1934) have recorded an incidence of 9 3 per cent in 535 autopsies in Vizagapatam

The incidence of hepatic cirrhosis in South India has been studied from an analysis of (1) the Annual Report of the Health Officer of the Corporation of Madras, (2) hospital admission registers and (3) post-mortem figures

The figures embodied in the Health Officer's report are, themselves, recorded by qualified medical men, ' the medical registrars' employed by the Corporation A certain amount of error is inevitable since certification of a death is not essential in every case, except in the case of hospital deaths

Table I shows the death rate from cirrhosis, per hundred thousand of population It will be noticed that, from the year 1929, a separate column is being maintained

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(827)

for biliary cirrhosis   This is largely owing to the high incidence of a special type, the infantile biliary cirrhosis, which is very fatal —

TABLE I

Year	Deaths from cirrhosis	Total deaths	Estimated population for mid year	Deaths per 100,000 of population
1923	124	19,933	528,680	23 5
1924	136	21,960	529,620	25 7
1925	195	25,000	532 503	36 6
1926	211	23,776	533,123	39 6
1927	184	22,364	534,629 (approximate)	34 4
1928	177	26,715	536,136	34 9
1929	(173+24) 197	22,415	537,149	36 7
1930	(170+13) 183	22,839	538,483	34 1
1931	(147+32) 179	23,163	647,230 (census)	27 7
1932	(187+23) 210	22,290	664,900	33 2
Average mortality from hepatic cirrhosis for ten years, per hundred thousand of population in Madras				32 6

A comparison of these figures with those of the Registrar-General for England and Wales for a period of nine years is of great interest —

TABLE II

Year	Deaths from cirrhosis	Estimated population	Deaths per 100,000 of population
1920	1,763	37,524,000	4 70
1921	1,927	37,897,000	5 80
1922	1,906	38,158 000	4 99
1923	1,774	38 402,000	4 62
1924	1,756	38,756,000	4 53
1925	1,847	38,890,000	4 75
1926	1,865	39,067,000	4 77
1927	1,860	39,290,000	4 73
1928	1,890	39,482,000	4 79
Average mortality in England and Wales from hepatic cirrhosis for nine years prior to 1929, per 100,000 of population			4 773

From a study of these figures it is evident that cirrhosis is nearly seven times as common here as in England and Wales, as judged by the mortality reports It is true that the Registrar-General's figures range higher prior to 1916, but even these are considerably smaller than the figures for Madras   It is also clear that this high incidence is not merely due to infantile biliary cirrhosis, since the available figures for Madras, of this type, are small

Hospital statistics are of great use in determining the possible ætiological factors   A study of the register of admissions to the Government Rayapuram Hospital, Madras, shows 183 cases of cirrhosis during the last three years   The total admissions amount to 30,817 cases   Six of these cases were of the infantile type, while the remaining 177 were of the portal type, some with an enlarged

palpable liver, while in others the liver had contracted, and ascites and other signs of decompensation had set in   Apart from these infantile types no case of biliary cirrhosis is recorded   Table III shows the distribution of portal cirrhosis according to race and caste —

TABLE III

Year	Hindus	Mohammedans	Europeans and Anglo-Indians	Other castes
1931	62	4	*Nil*	2
1932 .	44	2		
1933	57	1		5
Totals for 3 years	163	7	*Nil*	7
Percentages	92 10	3 95	*Nil*	3 95

Out of 1,819 Europeans and Anglo-Indians admitted to the hospital, there were no cases of cirrhosis   While it is no doubt true that arrack drinking is sometimes met with among the poorer class Hindus, it is forbidden by religion and usage among the Mohammedans   Beer and toddy drinking is common among the poorer class Anglo-Indians living in Madras and yet cirrhosis has a distribution that seems to be more on a population basis than on the incidence of alcoholism, as shown in Table IV —

TABLE IV

Showing the race distribution of the in-patients admitted to the Government Rayapuram Hospital, Madras, during the years 1931–1933

Year	Hindus	Mohammedans	Europeans and Anglo Indians	Other castes
1931	7,414	435	646	1,222
1932	8,018	527	579	1,203
1933	8,354	548	594	1,277
Totals for 3 years	23,786	1,510	1,819	3,702
Percentages	77 18	4 90	5 90	12 02

This question of a non-alcoholic cirrhosis in India has attracted a good deal of interest from tropical clinicians   Tucker (1908) ascribed it to malarial infection, while Rogers (1911) regarded amœbiasis as a possible cause

A study of the sex distribution of these 177 cases shows only 43 cases in women, while the rest were in men in a proportion of 1 3 35, while hospital admissions show a sex proportion of 1 2 01   Table V shows the age distribution —

TABLE V

Age	1931	1932	1933	Totals for 3 years
10-20	4	4	5	13
20-30	10	6	7	23
30-40	28	13	17	58
40-50	18	12	27	57
Above 50	8	10	6	26

Thus, though the frequency is greatest between the ages of 30 and 50, a definite proportion is met with in childhood and adolescence

Autopsy figures of the Madras General Hospital, as published in the Annual Hospital Reports, show that the incidence during recent years is low   Thus there are only 25 cases of cirrhosis out of 747 autopsies of clinical cases during the years 1927–1931   Post-mortem records of the Government Rayapuram Hospital, Madras, show a much higher incidence of 24 cases in 368 autopsies   This is partly because the hospital, situated near Washermanpet, serves to attract the poorer class of patients among whom cirrhosis seems to be more common in Madras   An infirmary is also attached to the hospital and serves as an asylum for incurables If autopsy figures from Madras are considered as a whole, we get an incidence of 4 39 per cent in 1,115 autopsies, a figure slightly lower than that of Rogers for Bengal and much lower than that of Tirumurti and Radhakrishna Rao for Vizagapatam, but decidedly higher than Berlin figures quoted by Rogers

When we take up the incidence of different ætiological types, the only figures that are available for South India are those published by one of us (Bhaskara Menon, 1934), from an analysis of autopsy records

Only cirrhosis with splenic enlargement was considered   Table VI shows these figures as compared with those of Tirumurti and Radhakrishna Rao (*loc cit*) for Vizagapatam —

TABLE VI

Authority	Totals	Portal	Capsular	Biliary	Banti's	Syphilis	With malaria	With kala azar
Bhaskara Menon (1934)	115	92		7	2	4	5	5
Tirumurti and Radhakrishna Rao (1934)	39	25	2	3	3	6	*Nil*	*Nil*

As a further study of these cases was not possible, we had to fall back on recent autopsies, where specimens were available and special histological methods could be applied   There were twenty-four of these cases   After a careful histological study of these cases, we adopted this provisional classification —

TABLE VII

Portal cirrhosis with fine granularity	5
Portal cirrhosis of the hob nail type with coarse nodules	11
Fatty cirrhotic liver	1
Toxic cirrhosis of Mallory with large and small nodules with extensive vascular tissue in the liver	1
Biliary cirrhosis (obstructive and infective)	*Nil*
Capsular cirrhosis, associated with malaria	3
Perihepatitis as part of a chronic peritonitis	1
Cirrhosis associated with kala azar	*Nil*
Syphilitic cirrhosis	1
Banti's type with extreme splenic enlargement and late cirrhosis	1
TOTAL number of cases of cirrhosis of the liver	24
TOTAL number of cases of acute yellow atrophy during the same period	3

DISCUSSION

With regard to the question of types of cirrhosis, Mallory's classification of portal cirrhosis into an alcoholic and a toxic type is of great interest   The 'toxic cirrhosis' of Mallory (1911), or the nodular hyperplasia of Marchand (1895), is characterized by the presence of large and small nodules with a collapsed vascular stroma in between   Extensive destruction of the liver-cells is followed by a condensation of the stroma rather than active proliferative growth, which Mallory considers is the essence of alcoholic cirrhosis   We would regard the process as one of sub-acute toxic hepatitis followed by regeneration, while the alcoholic type would be considered more chronic   In fact, the modern conception of cirrhosis as a chronic hepatitis has done much to clear the obscurity regarding the nature of the lesion   At one end of the series, we have acute hepatitis, ranging from mild catarrhal jaundice to the severe necrotic lesions of acute yellow atrophy   There are sub-acute types, which range in severity from sub-acute liver atrophy, the nodular hyperplasia of Marchand to the toxic cirrhosis of Mallory   At the other end, we have the chronic

types of the coarsely nodular and the granular livers where even now the causes are unknown, though alcohol was suspected   At the present day, the question of alcohol itself is undecided, even though it is argued that cirrhosis is less frequent with the introduction of prohibition in America   If this alcoholic type is regarded as toxic, the whole problem is one of severity of the toxic substance and the duration of the toxic process   The frequency of cirrhosis in the tropics would thus be capable of a simple explanation, even though alcoholism is certainly less frequent here   A similar explanation would suffice for the number of cases of acute yellow atrophy which occur apart from the toxæmias of pregnancy   From the figures we have quoted, we believe that the types of portal cirrhosis, that are met with here, are essentially the same as in Europe and America   The high incidence is not due to the frequency of any special toxic type described by Mallory   Prolonged intoxication, especially from the intestine, is a probable cause, since bowel diseases are so common in the tropics   This could possibly induce damage in the area of the distribution of the portal vein and the common hob-nail cirrhosis is a possible result   Alcohol cannot be regarded as a very important cause, at least in South India, where the race incidence and age periods show no definite relation to alcoholism   It is worthy of note that Tirumurti and Radhakrishna Rao (*loc cit*), from their analysis of cases in Vizagapatam, conclude that alcohol cannot be implicated

The dysenteric origin of portal cirrhosis has been put forward by Rogers (1911), who noticed a special association with amœbic dysentery   He argues that constant infection of the bowels with amœbæ from the intestine, in chronic amœbiasis, is followed by fibrosis around necrotic foci   He gives an instance of an amœbic abscess with early cirrhotic change around the abscess spreading to the liver   Two such cases are reported by Tirumurti and Radhakrishna Rao (*loc cit*)   While it is true that fibrotic changes with the formation of a wall is an occasional result, when the abscess gets encapsulated, such changes are not usual in amœbic abscesses   We have ourselves a specimen of a chronic encysted abscess on the under surface of the left lobe with irregular scars in the neighbourhood   The fibrosis, however, had not spread throughout the liver, but was confined to an area around the abscess   Regarding the association between dysentery and cirrhosis, one of us (B M) carried out an analysis of autopsy records in the Madras Medical College, while working in the pathology department in 1928   The results, which are now published for the first time, show that out of 119 cases of portal cirrhosis, 45 showed definite inflammatory lesions in the large intestine   These lesions could be classified into old pigmented scars, small hæmorrhagic ulcers, superficial ulceration with extensive congestion, extensive superficial ulceration involving large tracts of mucosa, large deep ulcers with necrotic base, etc   Mere congestion of the intestine with or without œdema, though often met with in bacillary dysentery, is not included, since portal decompensation might induce such changes   Out of these 119 cases, the intestines were not examined in three cases, so that the percentage works out as 38 87 of hepatic cirrhosis, showing dysenteric lesions   Out of these 45 cases showing dysenteric lesions, in 32 cases the lesions resembled those met with in acute and sub-acute bacillary dysentery   They were either superficial ulcers with extreme congestion, capillary hæmorrhages or small follicular ulcers surrounded by zones of congestion or extensive superficial ulceration involving large tracts of mucosa   The remaining 13 cases showed frank amœbic ulceration or healed pigmented scars   A comparison of these figures with the proportion of _dysenteric lesions, met with in hospital

autopsies in general, is of interest   Out of 368 recent autopsies in the Government Rayapuram Hospital, Madras, dysenteric lesions were found in 59 cases or 16 30 per cent   It seems, therefore, that there is some evidence for a positive association between dysenteric lesions in the intestines and portal cirrhosis   It is only by a more critical study of the intestinal lesions in cirrhosis that this problem can be settled

When we pass on to consider the next type, the biliary cirrhosis of adults, Rogers remarks that no authentic cases are recorded in Calcutta autopsies   One of us (Bhaskara Menon, *loc cit*) in an analysis of Madras autopsies has noticed definite obstructive types and possible infective types where the infection has apparently spread from the gall-bladder   Of biliary cirrhosis of infants, we have no recent instance in this series, since cases of this type seldom come up to the post-mortem table

Capsular cirrhosis, though emphasized by Chauffard (1902) in his classification, has met with very little comment   We believe that a distinction must be made between hyaloserositis involving the liver or the Zuckergussleber of Curschmann (1884), as it is called, and the true capsulitis, which often extends to the substance of the organ in definite bands in between the lobules   The cause of this condition is unknown, though syphilis has been suspected   We have in our series three cases of this capsular type of cirrhosis associated with malaria, where it is possible that repeated attacks with congestion and swelling of the liver were followed by a thickening of the capsule and a spreading fibrosis, similar to what is commonly found in the spleen   Externally, these livers look somewhat nodular with coarse nodules, but on section there is not the same extensive invasion of the substance of the organ as in the hob-nailed liver   Of perihepatitis, as part of an universal condition associated with ascites and chronic peritonitis, we have one instance, where the cause was obscure

Kala-azar cirrhosis, as described by Rogers (1911), seems to be a rarity here One of us has seen an instance of a portal type with kala-azar parasites in the Kupffer cells, but whether there is a direct association between the two conditions it is not possible to decide   Shanks and De (1931) have, in their histological studies, found no direct relationship   While enlargement of the liver is the rule in kala-azar, we see no reason why, in chronic cases, fibrotic changes may not occur, in view of the intimate relation between the reticulum cell and the fibrocyte   The rarity of the chronic type of kala-azar is a possible explanation why such cases are not commonly met with now

Splenic anæmia, with a late portal cirrhosis, forming the syndrome described by Banti, is distinctly uncommon and is responsible for only one of our cases, though Trimurti and Radhakrishna Rao (*loc cit*) consider it fairly frequent in Vizagapatam It seems to us that the division between this group and cases of portal cirrhosis with marked splenic enlargement is difficult, since the ' fibro-adenie ' of Banti is only a type of reticular increase which has nothing specially characteristic

Summary and conclusions

1   An analysis of mortality returns of Madras city, of hospital admissions in the Government Rayapuram Hospital, Madras, and of autopsy records of Madras

of recent years shows a much higher incidence of hepatic cirrhosis here than in Europe

2 A study of 177 hospital cases shows that cirrhosis has no special association with alcoholism   The race incidence is more or less in relation to the race proportion in the general population, as judged by the hospital admissions

3 A careful study of 24 specimens of hepatic cirrhosis has shown that the portal type is the most common, and that this is similar to the so-called alcoholic type met with in Europe and America   Mallory's ' toxic ' type, anatomically considered, is rare

4 From autopsy records, some positive evidence is brought forward to show that there is a possible relationship between portal cirrhosis and dysentery as emphasized by Rogers   But the evidence is more in favour of bacillary dysentery than amœbic   This may be capable of two interpretations   It may be that, in cirrhosis of the liver, the resistance of the intestine is so lowered that dysenteric lesions might occur as a complication or there may be a more direct ætiological relationship between dysentery and hepatic lesions

5 Cirrhosis of a capsular type has been recorded in three cases of malaria

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A NOTE ON SPLENIC ENLARGEMENT IN MALIGNANT HEPATOMA

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ROGERS (1925) in his Finlayson lecture was the first to draw attention to the unusually high incidence of primary carcinoma of the liver in Bengal He emphasized that this feature was in keeping with the much greater incidence of cirrhosis of the liver in Bengal Studies on the incidence of carcinoma of the liver in South India by Basu and Vasudevan (1929) showed a similar high incidence of primary, as compared with secondary growths, in a proportion of 1 45 1, whereas autopsy figures from Guy's hospital show a proportion of 1 24 Rolleston and McNee (1929) put down the incidence as varying between 1 20 and 1 40 Basu and Vasudevan hold that ' the germane factors are probably the frequency of liver cirrhosis and in respect of the ratio between primary and secondary growths, the relative infrequency of carcinoma of the bowel' The type of carcinoma described is the malignant hepatoma which is believed to arise as a sequence of coarse cirrhosis Many of the growths described are of the coarse nodular type, quite unlike the massive ' Cancer en amande ' of Hanot and Gilbert or of the diffuse infiltrating type Oncogenetically, hepatomas are tumours that arise from the hepatic cells, some are regarded as simple adenomata while others are distinctly malignant The transition between nodular hyperplasia, multiple adenomas and malignant hepatoma is often indistinct It has been argued that the same toxin that causes the cirrhosis may stimulate the liver-cells to such an extent that cirrhosis and carcinoma develop at the same time Others hold that carcinoma is the primary change, while cirrhosis is secondary and regarded as a tissue response Turnbull and Worthington (1908) have, however, described the sequence of changes in the transition from nodular hyperplasia to

(837)

multiple adenomas and carcinoma Rolleston and McNee (*loc cit*) hold that the history, the development and the morbid anatomy of these cases are more in favour of the view of a cirrhosis carcinomatosa In about half the cases, Rolleston and McNee have described splenic enlargement

In a recent study on the incidence of splenic enlargement in South India from autopsy records of the Madras Medical College and Rayapuram Medical School, one of us (Bhaskara Menon, 1934) was struck with the high proportion of cases of splenic enlargement in malignant hepatoma In going through the same series, recorded by Basu and Vasudevan (*loc cit*), splenic enlargement was met with in twenty-two cases out of twenty-nine cases of primary growth, i e , in 75 8 per cent In a previous paper one of us has pointed out that the enlargement is of the nature of a hyperplasia, similar to the condition met with in cirrhosis Secondary deposits were not met with, except in one case, where no histological examination was made as to the nature of the soft nodules in the spleen

If one were to assess the significance of the splenic enlargement in malignant hepatoma in India, one has to take into consideration the rarity of secondary growths in the spleen in carcinoma of the alimentary tract It would seem as if the splenic enlargement was not so much the result of the growth in the liver as the antecedent cirrhosis That the enlargement is not due to any co-existing tropical condition is borne out by the careful analysis previously referred to The frequency of primary carcinoma of the liver in India has been attributed to the much greater incidence of hepatic cirrhosis The co-existence of splenic enlargement in a large proportion of these cases would thus appear to emphasize this view It may also be argued that the sclerogenic agents that induce cirrhosis in India are possibly different in that they set up a degree of chronic irritation sufficient to induce malignant growth

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ON TYPES OF MENINGOCOCCI ISOLATED DURING THE 1934 EPIDEMIC OF CEREBRO-SPINAL MENINGITIS IN INDIA, WITH SPECIAL REFERENCE TO THE MANUFACTURE OF PROPHYLACTIC VACCINE

BY

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DURING the last few years there has been an increase in the incidence of cerebro-spinal fever in India   Russell (1933) has summarized the recorded cases in India for the years 1927 to 1932 and, in a subsequent communication to the Office Internationale d'Hygiene Publique, brought the information up to date by June 1934

Table I and the Chart show the recorded incidence of cerebro-spinal fever to the end of 1934   These figures are probably not complete but the general progress of the disease is shown by the fact that in the five years 1930 to 1934 the respective numbers of cases recorded was 23, 45, 174, 458 and 2,953

There has been a considerable increase in certain of the larger towns in India Ahmedabad City in the Bombay Presidency has suffered severely, first cases during the present epidemic being recorded in December 1933 and between that date and the end of June 1934 a total of 592 cases with 271 deaths was recorded

In Calcutta also there was a considerable incidence from 1932 to 1934—the total figures for the 3 years being respectively 145, 387 and 782

A severe outbreak in Delhi occurred in 1934 with 390 cases and 265 deaths

For the treatment of cerebro-spinal meningitis in India, the sera in use are mainly those imported from Europe and America, and in view of the possibility of continuance and extension of epidemic cerebro-spinal meningitis, it has been considered advisable to determine the types of meningococci responsible for the infection, in relation to the suitability of the sera used in treatment

Cerebro-Spinal Meningitis in India

TABLE I.

Incidence and mortality figures of cerebro-spinal fever cases for the years 1930 to 1934

(Monthly distribution of cases)

Year	JANY Cases	JANY Deaths	FEBY Cases	FEBY Deaths	MARCH Cases	MARCH Deaths	APRIL Cases	APRIL Deaths	MAY Cases	MAY Deaths	JUNE Cases	JUNE Deaths	JULY Cases	JULY Deaths	AUG Cases	AUG Deaths	SEPT Cases	SEPT Deaths	OCT Cases	OCT Deaths	NOV Cases	NOV Deaths	DEC Cases	DEC Deaths	TOTALS Cases	TOTALS Deaths
1930	2	1	1	1	4	2	0	0	1	0	2	2	2	3	0	0	3	3	1	1	2	1	5	1	23	15
1931	4	1	9	5	1	1	10	7	3	2	3	4	0	0	2	0	2	2	5	3	3	1	3	2	45	28
1932	0	0	10	8	19	12	23	19	7	7	9	8	20	18	17	17	8	7	24	24	12	12	25	26	174	158
1933	13	13	30	31	54	54	51	51	38	33	20	12	43	41	68	66	14	14	35	28	33	18	59	37	458	398
1934	101	74	228	138	622	300	546	324	383	255	138	105	157	110	158	120	130	98	113	69	166	75	211	125	2,953	1,793

The incidence of the disease in 1934 shows a very striking increase as compared with the previous years (The diagnosis in a large majority of these cases is based on clinical and epidemiological grounds)

CHART

Showing monthly incidence of cerebro-
spinal fever cases in India
for the years 1930-34
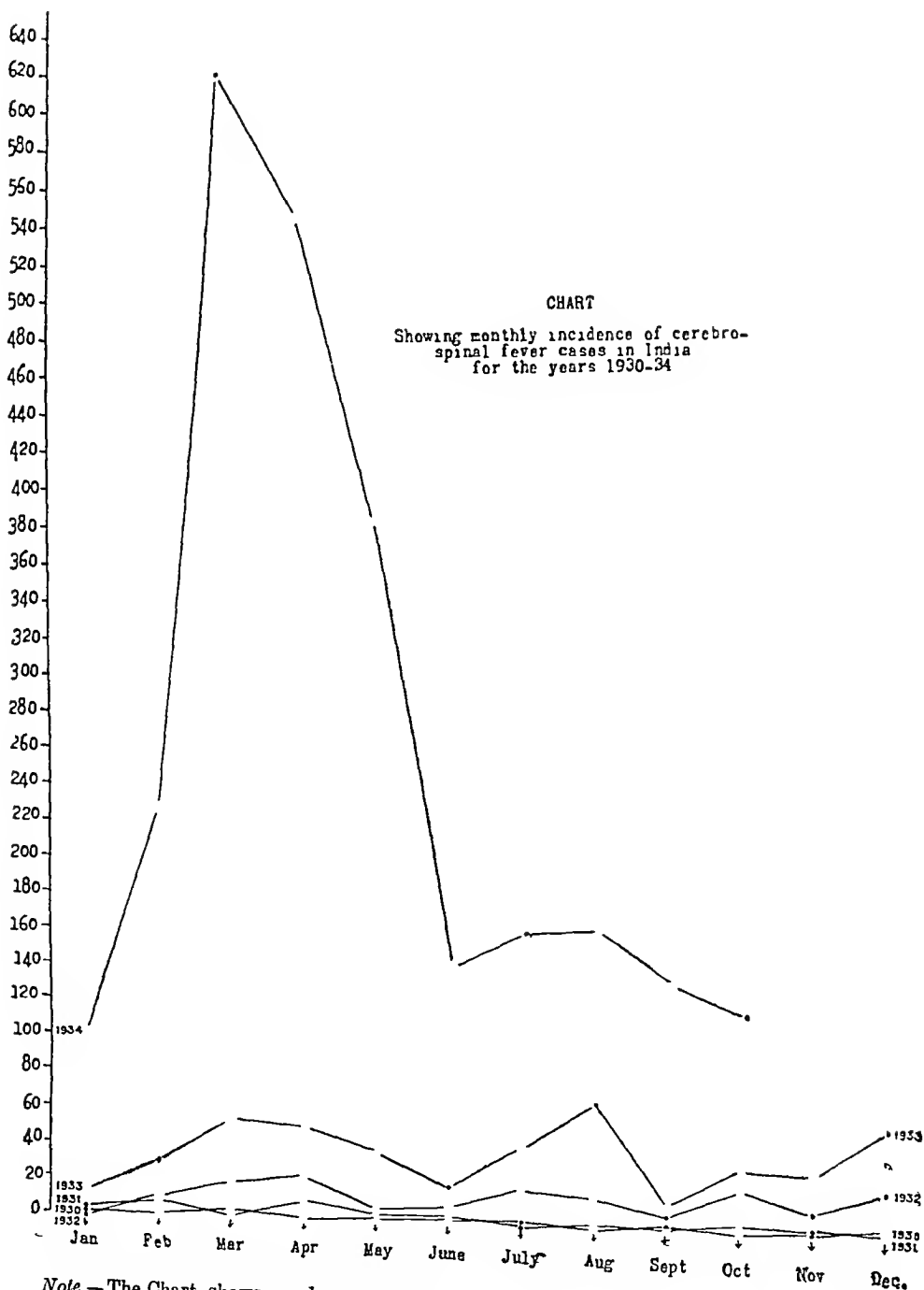
Note — The Chart shows incidence upto end of October 1934 only, but the peak again rose to
211 by the end of December 1934

An inquiry was accordingly carried out in Delhi during the epidemic season of 1934 for the purpose of obtaining strains for typing   The procedure adopted for culture was as follows —

CULTURAL METHODS

Cerebro-spinal fluid obtained by lumbar puncture was allowed to drop directly on to the surface of the following media in slopes

    (1) Ten per cent fresh pigeon blood in nutrient agar, pH 7 4

    (2) Dorset's egg medium

The tubes were warmed at 37°C for about two hours before sowing and were transferred immediately to the incubator   In acute cases a pure growth of meningococci was always obtained in 24 hours on pigeon-blood agar and Dorset's egg medium, and in sub-acute and chronic cases of over ten days' duration growth was only obtained on pigeon-blood agar in 48 hours   In only one case of a convalescent who had been in hospital for over a month a culture was not obtained   One per cent glucose agar, blood gelatine and semi-solid blood agar were also tried but gave less satisfactory results for primary culture

The strains were maintained by bi-weekly sub-cultures on pigeon-blood agar and reserve stock was kept on blood gelatine and semi-solid blood agar   The viability of the organism in the cerebro-spinal fluid and also in the primary culture on different media was tested by daily sub-culture on pigeon-blood agar   The survival period is shown in Table II —

TABLE II

Meningococcus, Delhi strain	LIFE IN DAYS AT 37°C					
	Cerebro-spinal fluid	Semi solid blood agar	Blood gela tine	Glucose agar	Pigeon blood agar	Dorset's egg
1	42	35	No growth	55	35	25
2	48	35	73	35	73	48
3	24	35	55	25	25	55
5	23	35	55	48	35	73
6	18	35	55	10	35	10
7	19	10	55	No growth	48	55
8	1	No growth	7	,,	4	No growth
9	1	,,	No growth	32	4	32
11	1	,,	,,	No growth	7	32

For isolation and maintenance of primary growth of the meningococcus 10 per cent fresh pigeon blood added to three per cent nutrient agar made with tryptic digest of mutton, pH 7 4, appeared to be the most satisfactory medium   We seldom failed to obtain growth on this medium   The colony appearance of meningococcus on this medium is of two types   (a) small, smooth, well-defined and raised colonies measuring 1 mm to 2 mm in diameter and (b) large, rather flat colonies with an irregular margin and a granular centre   After 48 hours the colonies showed a slightly buff tinge

TYPING OF STRAINS

The strains were tested against —

(a) Type sera received from the Standards Laboratories, Oxford

(b) Sera supplied by Dr W M Scott of the Pathological Laboratories of the Ministry of Health, London

Eighteen hours' growth was emulsified in 3 c c to 5 c c of sterile normal saline and heated at 65°C for one hour   Agglutination tests were carried out according to Dreyer's technique and results were read after four, and twenty-four hours

(a) Indian strains of meningococci tested against high titre sera supplied by Standards Laboratories, Oxford

Indian strains

Type sera	Delhi strains									Kasauli strain	Solon strain
	1	2	3	5	6	7	8	9	11	12	13
Type I	—	—	—	250	50	50	—	50	50	125	125
Type II	—	—	—	—	50	—	—	—	—	—	50
Type III	250	500	500	—	—	500	125	125	125	500	500
Type IV	—	—	—	—	—	—	—	—	—	—	—

(b) Indian strains of meningococci tested against high titre sera supplied by the Ministry of Health, London

Indian strains

Type sera	Delhi strains						Kasauli strain	Solon strain
	1	2	3	5	6	7	12	13
Type I Serum from Ministry of Health	50	—	125	250	50	125	125	125
Type III Serum from Ministry of Health	500	1,000	1,000	1,000	50	2,500	2,500	1,000

(c) High titre sera were prepared at Kasauli against the Indian strains of meningococci and tested against standard cultures of types I and III which were kindly supplied by Dr W M Scott   The results were as follows —

Standard type cultures of meningococci	High titre serum prepared against Delhi strains of meningococci					
	1	2	3	5	6	7
Type I	—	—	—	1,000	50	—
Type III	1,000	50	500	50	500	500

From the above serological tests it would appear that —

Meningococcus Delhi strain 1 is type III

,,   ,,   2   ,,   III

,,   ,,   3   ,,   III

,,   ,,   5   ,,   I

,,   ,,   6   ,,   I (? III)

,,   ,,   7   ,,   III

,,   ,,   8   ,,   III

,,   ,,   9   ,,   III

,,   ,,   11   ,,   III

,,   Kasauli   ,,   III

Solon   ,,   III

Cultures of some of these strains were sent to the Pathological Laboratory of the Ministry of Health, London, and Dr W M Scott reported on their types The strains tested by him were Delhi strains 1, 2, 3, 5, 6, 7, and Kasauli strain His findings confirmed ours in Kasauli

A number of strains isolated in the Bombay Presidency were also received from the Director, Haffkine Institute, Bombay, for typing and comparison with the strains isolated in Delhi   Of 19 strains examined 14 belonged to type III and 2 to type I   Three strains were agglutinated by type specific sera of I and III to an almost equal degree   They belonged to group I

Fermentation reactions of these strains were also tested   Eleven strains fermented glucose and maltose, 6 fermented maltose only, 2 fermented glucose only

It would appear that the epidemic strains of meningococci responsible for the outbreak of cerebro-spinal fever in India are of the same type as usually isolated in epidemic periods in other countries, i e, types I and III (group I), these being the types used for the manufacture of therapeutic serum ordinarily in use during epidemics

MENINGOCOCCUS VACCINE

The use of a prophylactic meningococcus vaccine has been suggested and such a vaccine has actually been employed in Turkey (Ismail, 1933, and Zernek, Klement and Bohuslav Feierabend, 1931)

In view of the difficulty of employing, in an adequate manner, serum treatment for a very large number of cerebro-spinal fever cases under Indian conditions the question of testing the value of such a vaccine has been considered   During the epidemic of 1934 a vaccine for the purpose was prepared at the Central Research Institute, Kasauli, and efforts were made to obtain a trial under conditions which would permit of estimating its value

METHOD OF MANUFACTURE OF PROPHYLACTIC MENINGOCOCCAL VACCINE

Media —Dorset's egg and pigeon-blood agar we found to be unsuitable for preparation of large quantities of vaccine   One per cent glucose agar (pH 7 4) yielded excellent growth and this we eventually adopted as the medium for bulk manufacture

Selection of strains —Freshly isolated strains of meningococci from acute, sub-acute, chronic and convalescent cases were used as the seed   For description of the types of cases from which they were obtained *see* Table III   The majority of the strains were type III and some of type I   The vaccine is either the third or fourth sub-culture of primary growth

TABLE III

Cases	Date of onset	Date of admission	Number of lumbar punctures performed before taking C S F for culture and amount of serum given	Date of taking C S F for culture	Macroscopic and microscopic appearance of cerebro spinal fluid	Condition of the patient	Result
1 (U)		24-3-34	Nil	24-3-34	Turbid Pressure+++ Polymorphs, Gram-negative and cocci present	Marked retraction of the neck Kernig's sign positive Delirious	Cured Discharged 20-4-34
2 (U K)		20-9-34	Punctured only 2 hours before taking C S F 20 c c serum given	21-9-34	Slightly turbid Pressure slight Pus cells and few Gram negative cocci present	Admitted in a comatose condition Marked retraction of the neck Kernig's sign present	Died 21-9-34
3 (S S)	21-3-34	24-3-34	Nil	25-3-34	Turbid and under great pressure Pus cells and Gram negative cocci present	Delirious and violent Kernig's sign positive	Died 30-4-34
4 (B J)	19-3-34	23-3-34	L P 1, serum nil	24-3-34	Turbid Pressure +++ Pus cells and Gram-negative cocci present	Comatose Marked retraction of the neck Kernig's sign positive	Died 27-3-34
5 (A U K)	19-3-34	21-3-34	L P 2, serum nil	24-3-34	Very turbid Pressure +++ Pus cells and Gram-negative cocci present	Marked retraction of the neck Kernig's sign positive	Cured Discharged 20-4-34
6 (S L)	19-3-34	23-3-34	L P 2, serum 20 c c	24-3-34	Turbid Pressure +++ Gram-negative cocci present	do	Cured Discharged 12-4-34
1 (S R)	18-3-34	20-3-34	L P 4, serum 20 c c	24-3-34	Turbid Pressure slight Pus cells and Gram-negative cocci present	do	Cured Discharged 12-4-34
1 (M)	17-8-34	21-8-34	L P 23, serum 160 c c	20-9-34	Very slight turbidity Pressure slight Gram-negative cocci present	Comatose Marked retraction of the neck Kernig's sign positive	Left hospital 21-9-34
2 (I H)	1-9-34	4-9-34	L P 13, serum 130 c c	21-9-34	C S F clear No pressure Few pus cells No Gram-negative cocci seen	Rigidity of the neck Kernig's sign positive Patient semiconscious	Not known

Preparation of seed material and vaccine

Sub-cultures from the primary growth are made on pigeon-blood agar   After 24 hours the growth is tested for purity   If satisfactory, 100 c c of Malone's (1921) influenza broth are inoculated with this culture   Next day the broth culture is tested for purity and if satisfactory, 4 c c are inoculated into each of a batch of whisky bottles containing a rolled medium of 1 per cent glucose agar in tryptic digest of mutton at pH 7 4, each bottle containing 100 c c of medium   An equal number of bottles is inoculated with each strain   The bottles are rolled to spread the inoculum over the surface of the medium and incubated at 37°C for 48 hours, when the growth from each bottle is washed off with 20 c c of sterile normal saline kept warm at 37°C before use   The growth from each bottle is poured into a separate sterile test-tube and a sample taken for test of purity of growth   The tubes are heated at 56°C for half an hour and 0 1 c c of carbolic acid added to each tube for temporary preservation   Next day, when the purity of growth from the bottles has been confirmed, contents of 20 tubes representing an equal number of different strains are pooled into one-litre flasks and enough saline is added to bring the volume to 500 c c

The contents of each flask are now standardized by means of Brown's opacity tubes and carbolic acid is added to bring the strength of antiseptic in the concentrated vaccine to 1 per cent   The flasks are left at room temperature for 48 hours at the end of which period sterility and toxicity tests are carried out

(a) Sterility is tested both aerobically and anaerobically on pigeon-blood agar and glucose broth

(b) Toxicity is tested by injecting subcutaneously 1 c c of the concentrated vaccine into a guinea-pig of 350 grammes weight   If the animal shows little reaction and is alive and well at the end of ten days the vaccine is passed

The flasks of concentrated vaccine are stored at room temperature in the dark until required for use   The vaccine is issued in a strength of 1,000 million organisms per c c with 0 5 per cent phenol and two or preferably three doses are recommended, 0 5, c c, 1 c c and 2 c c to be injected subcutaneously at weekly intervals   In the preliminary work on the vaccine its toxicity was tested on a number of *rhesus* monkeys weighing 3 lb to 4 lb each, by injecting them subcutaneously with 4 c c and 8 c c of the stock vaccine   No marked general reaction was observed except a slight rise of temperature lasting 48 hours in some of the monkeys   Local reaction was not marked   A number of human volunteers were given three doses of the vaccine without showing any marked local or general reaction   As the result of these tests it was considered that the vaccine could safely be issued for use   Up to the end of December 1934, 32,853 c c of this vaccine have been issued   As regards the protective value of this vaccine the figures will not be available for another six to eight months at the end of which time it is hoped to issue a statement on the results of this method of prophylaxis

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NOTICE.

The following has been received for announcement —

Editor

' THE Tomarkin Foundation is organizing, under the auspices of the University of Brussels, its VIIth International Medical Post-Graduate Congress officially approved by the General Commissariat of the Government for the World Exhibition

This Congress dealing with ' Diagnostic and Therapeutic Actualities ' will be held during the World Exhibition from the 12th to 19th September next in the Faculty of Medicine of the University in Brussels and from the 20th September to the 2nd October at Spa

More than 100 professors from nearly 45 Universities will lecture over the following special branches —

> Affections of the Cardio-Vascular System, Diseases of the Blood, Cancer, Tropical Diseases, Infectious Diseases, Free Lecture Field and Social Medicine

For all particulars please apply to the Secretary, Tomarkin Foundation, Faculté de Médicine, 97, rue aux Laines, Bruxelles (Belgique) '

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